(19) Europäisch s Patentamt
European Pat nt Office
Office europ ´ n des brev ts

(11) EP 0 242 236 B2

(12) **NEW EUROPEAN PATENT SPECIFICATION**

(45) Date of publication and mention of the opposition decision:
21.08.1996 Bulletin 1996/34

(45) Mention of the grant of the patent:
10.10.1990 Bulletin 1990/41

(21) Application number: **87400141.5**

(22) Date of filing: **21.01.1987**

(51) Int Cl.[6]: **C12N 15/82**, A01H 1/00, C12N 5/10, A01H 5/10 // C12N9/10

(54) **Plant cells resistant to glutamine synthetase inhibitors, made by genetic engineering**

Durch Gentechnologie erhaltene und gegen Glutaminsynthetase-Inhibitoren resistente Pflanzenzellen

Cellules végétales résistantes aux inhibiteurs de la synthétase de glutamine, produites par génie génétique

(84) Designated Contracting States:
AT BE CH DE ES FR GB GR IT LI LU NL SE

(30) Priority: **11.03.1986 EP 86400521**

(43) Date of publication of application:
21.10.1987 Bulletin 1987/43

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Description

The invention relates to a process for protecting plant cells and plants against the action of glutamine synthetase inhibitors.

It also relates to applications of such process, particularly to the development of herbicide resistance into determined plants.

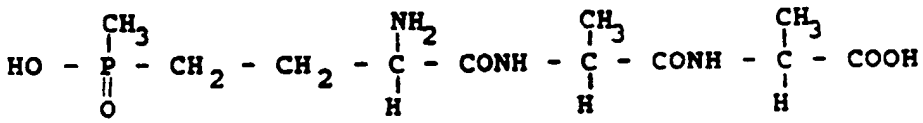
It relates further to non-biologically transformed plant cells and plants displaying resistance to glutamine synthetase inhibitors as well as to suitable DNA fragments and recombinants containing necleotide sequences encoding resistance to glutamine synthetase inhibitors.

Glutamine synthetase (hereinafter simply designated by GS) constitutes in most plants one of the essential enzymes of the development and life of plant cells. It is known that GS converts glutamate into glutamine. GS is involved in an efficient pathway (the only one known nowadays) in most plants for the detoxification of ammonia released by nitrate reduction, aminoacid degradation or photorespiration. Therefore potent inhibitors of GS are very toxic to plant cells. A particular class of herbicides has been developed, based on the toxic effect due to inhibition of GS in plants. These herbicides comprise as active ingredient a GS inhibitor.

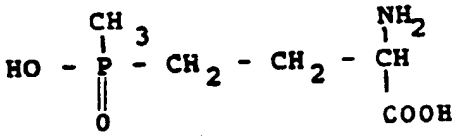
There are at least two possible ways which might lead to plants resistant to the inhibitors of the action of glutamine synthetase; (1) by changing the target. It can be envisaged that mutations in the GS enzyme can lead to insensitivity towards the herbidide; (2) by inactivation of the herbicide. Breakdown or modification of the herbicide inside the plant could lead to resistance.

Bialphos and phosphinothricin (hereinafter simply designated by PPT) are two such inhibitors of the action of GS, (ref. 16, 17) and have been shown to possess excellent herbidicial properties (see more particularly ref. 2 as concerns Bialaphos).

Bialaphos has the following formula (I):



PPT has the following formula (II):



Thus the structural difference between PPT and Bialaphos resides in the absence of two alanine aminoacids in the case of PPT.

These two herbicides are non selective. They inhibit growth of all the different species of plants present on the soil, accordingly cause their total destruction.

Bialaphos was first disclosed as having antibiotic properties, which enabled it to be used as a pesticide or a fungicide. Bialaphos can be produced according to the process disclosed in United States patent n° 3 832 394, assigned to MEIJI SEIKA KAISHA LTD. It comprises cultivating *Streptomyces hygroscopicus,* such as the strain available at the American Type Culture Collection, under the ATCC number 21,075, an recovering Bialaphos from its culture medium. However, other strains, such as *Streptomyces viridochromogenes,* also produce this compound (ref. 1).

Other tripeptide antibiotics which contain a PPT moiety are or might be discovered in nature as well, e.g. phosalacin (ref. 15).

PPT is also obtained by chemical synthesis and is commercially distributed by the industrial Company HOECHST.

A number of Streptomyces species have been disclosed which produce highly active antibiotics which are known to incapacitate procaryotic cell functions or enzymes. The Streptomyces species which produce these antibiotics would themselves be destroyed if they had not a self defence mechanism against these antibiotics. This self defence mechanism has been found in several instances to comprise an enzyme capable of inhibiting the antibiotic effect, thus of avoiding autotoxicity for the Streptomyces species concemed. This modification is generally reversed when the molecule is exported from the cell.

The existence of a gene which encodes an enzyme able to modify the antibiotic so as to inhibit the antibiotic effect against the host has been demonstrated in several Streptomyces producing antibiotics, for example, in *S. fradiae, S. azureus, S. vinaceus, S. erythreus,* producing neomycin, thiostrepton, viomycin and MLS (Macrolide Lincosamide

Streptogramin) antibiotics respectively (ref. 4) (ref. 5), (ref. 6), (ref. 14 by CHATER et al., 1982 describes standard techniques which can be used for bringing these effects to light).

In accordance with the present invention, it has been found that *Steptomyces hygroscopicus* ATCC 21,705, also possesses a gene encoding an enzyme responsible of the inactivation of the antibiotic properties of Bialaphos. Experiments carried out by the applicants have lead to the isolation of such a gene and its use in a process for controlling the action of GS inhibitors, based on PPT or derived products.

An object of the invention is to provide a new process for controlling the action in plant cells and plants of GS inhibitors.

Another object of the invention is to provide DNA fragments and DNA ecombinants, particularly modified vectors containing said DNA fragments, which DNA fragments contain nucleotide sequences able, when incorporated in plant cells and plants, to protect them against the action of GS inhibitors.

A further object of the invention is to provide non-biologically transformed plant cells and plants capable of neutralizing or inactivating GS inhibitors.

A further object of the invention is to provide a process for selectively protecting plant species against herbicides of a GS inhibitor type.

More specifically an object of the invention is to provide a NDA fragment transferable to plant cells- and to whole plants- capable of protecting them against the herbicidal effects of Bialaphos and of structurally analogous herbicides.

A further object of the invention is to provide plant cells resistant to the products of the class examplified by Bialaphos, which products possess the PPT unit in their structure.

The process according to the invention for controlling the action in plant cells and plants of a GS inhibitor when contacted therewith, comprises providing said plant with a heterologous DNA fragment including a foreign nucleotide sequence, capable of being expressed in the form of a protein in said plant cells and plants, under condition such as to cause said heterologous DNA fragment to be integrated stably through generations in the cells of said plants, and wherein said protein has an enzymatic activity capable of causing inactivation or neutralization of said glutamine synthetase inhibitor.

A preferred DNA fragment is one derived from an antibiotic-producing-Streptomyces strain (or a sequence comprising a nucleotide sequence encoding the same activity) and which encodes resistance to said GS inhibitors.

Preferred nucleotide sequences for use in this invention encode a protein which has acetyl transferase activity with respect to said GS inhibitors.

A most preferred DNA fragment according to the invention comprises a nucleotide sequence coding for a polypeptide having a PPT acetyl transferase activity.

A particular DNA fragment according to the invention, for the subsequent transformation of plant cells consists of a nucleotide sequence coding for at least part of a polypeptide having the following sequence:

X SER PRO GLU

183
ARG ARG PRO ALA ASP ILE ARG ARG ALA THR GLU ALA ASP MET PRO
228
ALA VAL CYS THR ILE VAL ASN HIS TYR ILE GLU THR SER THR VAL
273
ASN PHE ARG THR GLU PRO GLN GLU PRO GLN GLU TRP THR ASP ASP
318
LEU VAL ARG LEU ARG GLU ARG TYR PRO TRP LEU VAL ALA GLU VAL
363
ASP GLY GLU VAL ALA GLY ILE ALA TYR ALA GLY PRO TRP LYS ALA
408
ARG ASN ALA TYR ASP TRP THR ALA GLU SER THR VAL TYR VAL SER
453
PRO ARG HIS GLN ARG THR GLY LEU GLY SER THR LEU TYR THR HIS
498
LEU LEU LYS SER LEU GLU ALA GLN GLY PHE LYS SER VAL VAL ALA
543
VAL ILE GLY LEU PRO ASN ASP PRO SER VAL ARG MET HIS GLU ALA
588
LEU GLY TYR ALA PRO ARG GLY MET LEU ARG ALA ALA GLY PHE LYS
633
HIS GLY ASN TRP HIS ASP VAL GLY PHE TRP GLN LEU ASP PHE SER
678
LEU PRO VAL PRO PRO ARG PRO VAL LEU PRO VAL THR GLU ILE
723

in which X represents MET and VAL, which part of said polypeptide is of sufficient length to confer protection against Bialaphos to plant cells, when incorporated genetically and expressed therein, i.e as termed hereafter "plant-protecting capability" against Bialaphos.

A preferred DNA fragment consists of the following nucleotide sequence:

```
                                                        GTG AGC CCA GAA
     183
CGA CGC CCG GCC GAC ATC CGC CGT GCC ACC GAG GCG GAC ATG CCG
     228
GCG GTC TGC ACC ATC GTC AAC CAC TAC ATC GAG ACA AGC ACG GTC
     273
AAC TTC CGT ACC GAG CCG CAG GAA CCG CAG GAG TGG ACG GAC GAC
     318
CTC GTC CGT CTG CGG GAG CGC TAT CCC TGG CTC GTC GCC GAG GTG
     363
GAC GGC GAG GTC GCC GGC ATC GCC TAC GCG GGC CCC TGG AAG GCA
     409
CGC AAC GCC TAC GAC TGG ACG GCC GAG TCG ACC GTG TAC GTC TCC
     453
CCC CGC CAC CAG CGG ACG GGA CTG GGC TCC ACG CTC TAC ACC CAC
     498
CTG CTG AAG TCC CTG GAG GCA CAG GGC TTC AAG AGC GTG GTC GCT
     543
GTC ATC GGG CTG CCC AAC GAC CCG AGC GTG CGC ATG CAC GAG GCG
     588
CTC GGA TAT GCC CCC CGC GGC ATG CTG CGG GCG GCC GGC TTC AAG
     633
CAC GGG AAC TGG CAT GAC GTG GGT TTC TGG CAG CTG GAC TTC AGC
     678
CTG CCG GTA CCG CCC CGT CCG GTC CTG CCC GTC ACC GAG ATC
     723
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or of a part thereof expressing a polypeptide having plant-protecting capability against Bialaphos.

The invention also reltes to any DNA fragment differing from the preferred one indicated hereabove by the replacement of any of its nucleotides by others, yet without modifying the genetic information of the preferred DNA sequence mentioned hereabove (normally within the meaning of the universal genetic code), and furthermore to any equivalent DNA sequence which would encode a polypeptide having the same properties, particularly a Bialaphos-resistance-activity.

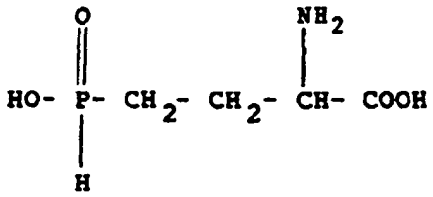
It will be understood that the man skilled in the art should be capable of readily assessing those pats of the nucleotide sequences that coould be removed from either side of any of the DNA fragments according to the invention, for instance by removing terminal parts on either sie of said DNA fragment, such as by an exonucleolytic enzyme, for instance Ba131, by recloning the remaining fragment in a suitable plasmid and by assaying the capacity of the modified plasmid to transform appropriate cells and to protect it against the Bialaphos antibiotic or herbicide as disclosed later, whichever assay is appropriate.

For the easiness of language, these DNA fragments will be termed hereinafter as "Bialaphos-resistance DNA", In a similar manner, the corresponding polypeptide will be termed as "Bialaphos-resistance enzyme".

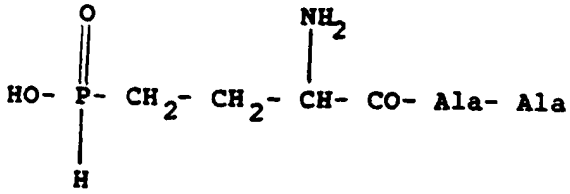
While in the preceding discussion particular emphasis has been put on DNA fragments capable, when introduced into plant cells and plants, to confer on them protection against Bialaphos or PPT, it should be understood that the invention should in no way be deemed as limited thereto.

In a same manner, the invention pertains to DNA fragments which, when introduced into such plant cells, would also confer on them a protection against other GS inhibitors, for instance of intermediate products involved in the natural biosynthesis of phosphonitricin, such as the compounds designated by the abbreviations MP101 (III), MP102

(IV), the formula of which are indicated hereafter:



(III)



(IV)

More generally, the invention has opened the route to the production of DNA fragments which, upon proper incorporation into plant cells and plants, can protect them against GS inhibitors when contacted therewith, as this will be shown in a detailed manner in relation to Bialaphos and PPT in the examples which will follow.

This having been established, it will be appreciated that any fragment encoding an enzymatic activity which would protect plant cells and plants against said GS inhibitors should be viewed as an equivalent of the preferred fragments which have been disclosed hereabove. This would apply especially to any DNA fragments that would result from genetic screening of the genomic DNAs of strains, particularly of antibiotic-producing strains, likely to possess genes which, eventhough structurally different, would encode similar activity with respect to Bialaphos or PPT, or even with respect to other GS inhibitors. One might envisage similar genes in other strains producing a PPT derivative.

Therefore, it should be understood that the language "Bialaphos-resistance DNA" or "Bialaphos-resistance enzyme" used thereafter as a matter of convenience is intended to relate not only to the DNAs and enzymes specifically concerned with resistance to PPT or most directly related derivatives, but more generally with other DNAs and enzymes which would be capable, under the same circumstances, of controlling the action in plants of GS inhibitors.

The invention also relates to DNA recombinants containing the above defined Bialaphos-resistance DNA fragments recombined with heterologous DNA, said heterologous DNA containing regulation elements and said Bialaphos-resistance DNA being under the control of said regulation elements in such manner as to be expressible in a foreign cellular environment compatible with said regulation elements.

By "heterologous DNA" is meant a DNA of an other origin than that from which said Bialaphos-resistance-DNA originated, e.g. is different from that of a *Streptomyces hygroscopicus* or *Streptomyces viridochromogenes* or even more preferably a DNA foreign to Streptomyces DNA. Particularly said regulation elements are those which are capable of controlling the transciption and translation of DNA sequences normally associated with them in said foreign environment. "Cellular" refers both to micro-organisms and to cell cultures.

This heterologous DNA may be a bacterial DNA, particularly when it is desired to produce a large amount of the recombinant DNA, such as for amplification purposes. In that respect a preferred heterologous DNA consists of DNA of *E. coli* or of DNA compatible with *E. coli.* It may be DNA of the same origin as that of the cells concerned or other DNA, for instance viral or plasmidic DNA known as capable of replicating in the cells concerned.

Preferred recombinant DNA contains heterologous DNA compatible with plant cells, particularly Ti-plasmid DNA.

Particularly preferred recombinants are those which contain GS inhibitor inactivating DNA under the control of a promoter recognized by plant cells, particularly those plant cells on which inactivation of GS inhibitors is to be conferred.

Preferred recombinants according to the invention further relate to modified vectors, particularly plasmids, containing said GS-inhibitor-inactivating DNA so positioned with respect to regulation elements, including particularly promoter elements, that they enable said GS inhibitor-inactivating DNA to be transcribed and translated in the cellular environment which is compatible with said heterologous DNA. Advantageous vectors are those so engineered as to cause stable incorporation of said GS inhibitor-inactivating DNA in foreign cells, particularly in their genomic DNA. Preferred modified vectors are those which enable the stable transformation of plant cells and which conver to the corresponding cells, the capability of inactivating GS inhibitors.

It seems that, as described later, the initiation codon of the Bialaphos-resistance-gene of the *Streptomyces hygroscopicus* strain used herein is a GTG codon. But in preferred recombinant DNAs of vectors, the Bialaphos-resistance-gene is modified by substitution of an ATG initiation codon for the initiation codon GTG, which ATG enables translation initiation in plant cells.

In the example which follows, the plant promoter sequence which has been used was constituted by a promoter

of the 35 S cauliflower mosaic virus. Needless to say that the man skilled in the art will be capable of selecting other plant promoters, when more appropriate in relation to the plant species concerned.

According to another preferred embodiment of the invention, particularly when it is desired to achieve transport of the enzyme encoded by the Bialaphos-resistance-DNA into the chloroplasts, the heterologous DNA fragment is fused to a gene or DNA fragment encoding a transit peptide, said last mentioned fragment being then intercalated between the GS inhibitor inactivating gene and the plant promoter selected.

As concerns means capable of achieving such constructions, reference can be made to the following British applications 84 32757 filed on December 28, 1984, and 85 00336 filed on January 7, 1985 and to the related applications filed in the United States of America (n° 755,173, filed on July 15, 1985), in the European Patent Office (n° 85 402596.2, (EP-A-189707) filed on December 20, 1985) in Japan (n° 299 730, filed on December 27, 1985), in Israel (n° 77 466 filed on December 27, 1985) and in Australia (n° 5 165 485, filed on December 24, 1985).

Reference can also be made to the scientific literature, particularly to the following articles:

VAN DEN BROECK et al., 1985, Nature, 313, 358-363;
SCHREIER and al., Embo, J., vol. 4, n° 1, 25-32.
These articles are also incorporated herein by reference.

For the sake of the record, be it recalled here that under the expression "transit peptide", one refers to a polypeptide fragment which is normally associated with a chloroplast protein or a chloroplast protein subunit in a precursor protein encoded by plant cell nuclear DNA. The transit peptide then separates from the chloroplast protein or is proteolytically removed, during the translocation process of the latter protein into the chloroplasts. Examples of suitable transit peptides are those associated with the small subunit of ribulose-1,5 bisphosphate (RuBP) carboxylase or that associated with the chlorophyl a/b binding proteins.

There is thus provided DNA fragments and DNA recombinants which are suitable for use in the process defined hereafter.

More particularly the invention also relates to a process, which can be generally defined as a process for producing plants and reproduction material of said plants including a heterologous genetic material stably integrated therein and capable of being expressed in said plants or reproduction material in the form of a protein capable of inactivating or neutralizing the activity of a glutamine synthetase-inhibitor, comprising the non biological steps of producing plant cells or plant tissue including said heterologous genetic material from starting plant cells or plant tissue not able to express that inhibiting or neutralizing activity, regenerating plants or reproduction material of said plants or both from said plant cells or plant tissue including said genetic material and optionally, biologically replicating said last mentioned plants or reproduction material or both, wherein said non-biological steps of producing said plant cells or plant tissue including said heterologous genetic material, comprises transforming said starting plant cells or plant tissue with a DNA-recombinant containing a nucleotide sequence encoding said protein, as well as the regulatory elements selected among those which are capable of enabling the expression of said nucleotide sequence in said plant cells or plant tissue, and to cause the stable integration of said nucleotide sequence in said plant cells and tissue, as well as in the plant and reproduction material processed therefrom throughout generations.

The invention also relates to the cells cultures containing Bialaphos-resistance-DNA, or more generally said GS-inhibitor-inactivating DNA, which cell cultures have the property of being resistant to a composition containing a GS inhibitor, when cultured in a medium containing a such composition at dosages which would be destructive for non transformed cells.

The invention concerns more particularly those plant cells or cell cultures in which the Bialaphos-resistance DNA is stably integrated and which remains present over successive generations of said plant cells. Thus the resistance to a GS inhibitor, more particularly Bialaphos or PPT, can also be considered as a way of characterizing the plant cells of this invention.

Optionally one may also resort to hybridization experiments between the genomic DNA obtained from said plant cells with a probe containing a GS inhibitor inactivating DNA sequence.

More generally the invention relates to plant cells, reproduction material, particularly seeds, as well as plants containing a foreign or heterologous DNA fragment stably integrated in their respective genomic DNAs, said fragments being transferred throughout generations of such plant cells, reproduction material, seeds and plants, wherein said DNA fragment encodes a protein inducing a non-variety-specific enzymatic activity capable of inactivating or neutralizing GS inhibitors, particularly Bialaphos and PPT, more particularly to confer on said plant cells, reproduction material, seeds and plants a corresponding non-variety-specific phenotype of resistance to GS inhibitors.

"Non-variety-specific" enzymatic activity or phenotype aims at referring to the fact that they are not characteristic of specific plant genes or species as this will be illustrated in a non-limitative way by the examples which will follow. They are induced in said plant materials by essentially non-biological processes applicable to the plants belonging to species normally unrelated with one another and comprising the incorporation into said plant material of heterologous

DNA, e.g. bacterial DNA or chemically synthesized DNA, which does not normally occur in said plant material or which normally cannot be incorporated therein by natural breeding processes, and which yet confers a common phenotype (e.g. herbicide resistance) to them.

The invention is of particular advantageous use in processes of protecting field-cultivated plant species against weeds, which processes comprise the step of treating the field with an herbicide, e.g. Bialaphos or PPT in a dosage effective to kill said weeds, wherein the cultivated plant species then contain in their genome a DNA fragment encoding a protein having an enzymatic activity capable of neutralizing or inactivating said GS inhibitor.

By way of illustration only, effective doses for use in the abovesaid process range from about 0.4 to about 1.6 KgIHectare of Bialaphos of PPT.

There follows now a disclosure of how the preferred DNA fragment described hereabove was isolated starting from the *Streptomyces hygroscopicus* strain available at the American Type Culture Collection under deposition number ATCC 21 705, by way of examplification only.

The following disclosure also provides the technique which can be applied to other strains producing compounds with a PPT moiety.

The disclosure will then be completed with the description of the insertion of a preferred DNA fragment conferring to the transformed cells the capability of inactivating Bialaphos and PPT. Thus the Bialaphos-inactivating-DNA fragment designated thereafter Bialaphos-resistance gene of "sfr" gene, isolated by the above described technique into plasmids which can be used for transforming plant cells and conferring to them a resistance against Bialaphos, also merely by way of example for non-limitative illustration purposes.

The following dislosure is made with reference to the drawings in which:

Fig. 1 is a restriction map of a plasmid containing a *streptomyces hygroscopicus* DNA fragment encoding Bialaphos-resistance, which plasmid, designated hereafter as pBG1 has been constructed according to the disclosure which follows;

Fig. 2 shows the nucleotide sequence of a smaller fragment obtained from pBG1, subcloned into another plasmid (pBG39) and containing the resistance gene;

Fig. 3 shows the construction of a series of plasmids given by way of example, which plasmids aim at providing suitable adaptation means for the insertion therein of the Bialaphos-resistance gene or "sfr" gene;

Fig. 4A and 4B show the construction of a series of plasmids given by way of example, which plasmids contain suitable plant cell promoter sequences able to initiate transcription and expression of the foreign gene inserted under their control into said plasmids;

Fig. 5A shows a determined fragment of the nucleotide sequence of the plasmid obtained in Fig. 3;

Fig. 5B shows the reconstruction of the first condons of a Bialaphos-resistance gene, from a *FokI/Bg 1*II fragment obtained from pBG39 and the substitution of an ATG initiation codon for the GTG initiation codon of the natural "sfr" gene;

Fig. 5C shows the reconstruction of the entire "sfr" gene, namely the last codons thereof, and its insertion into a plasmid obtained in Figs. 4A and 4B;

Fig. 6A shows an expression vector containing the "sfr" gene placed under the control of a plant cell promoter;

Fig. 6B shows another expression vector derived from the one shown in Fig. 6A, by the substitution of some nucleotides.

Fig. 7 shows the construction of a series of plasmids given by way of examples, to ultimately produce plasmids containing the promoter region and the transit peptide sequence of a determined plant cell gene, for the insertion of the "sfr" gene under the control of said promoter region and downstream of said transit peptide sequence.

The following experiment was set up to isolate a Bialaphos-resistance-gene from S. *hygroscopicus,* according to standard techniques for cloning into *Streptomyces.*

2.5 µg of *S. hygroscopicus* genomic DNA and 0.5 µg of *Streptomyces* vector pIJ61 were cleaved with *Psi*I according to the method described in ref. 6. The vector fragments and genomic fragments were mixed and ligated (4 hours at 10°C followed by 72 hours at 4°C in ligation salts which contain 66 mM Tris-HCl (ph 7.5), 1 mM EDTA, 10 mM $MgCL_2$, 10 mM 2-mercaptoethanol and 0.1 mM ATP) at a total DNA concentration of 40 µg ml^{-1} with T4 DNA ligase. Ligation products were introduced into 3×10^9 *S. lividans* strain 66 protoplasts by a transformation procedure mediated by polyethylene-glycol (PEG) as described hereinafter. These protoplasts gave rise to 5×10^7 colonies and 4×10^4 pocks after regeneration on 20 plates of R2 agar containing 0.5% of Difco yeast extract (R2 YE). Preparation and composition of the different mediums and buffers used in the disclosed experiments are described hereinafter. When these lawns were replica-plated on minimal medium plates containing 50 µg ml^{-1} Bialaphos, drug resistant colonies appeared at a frequency of 1 per 10^4 transformants. After purification of the drug resistant colonies their plasmid DNA was isolated and used to retransform *S. lividans* protoplasts. Non selective regeneration followed by replication to Bialaphos-containing-medium demonstrated at 100% correlation between pocks and Bialaphos resistant

growth. The recombinant plasmids of several resistant clones all contained a 1.7 Kb *PstI* insert (see Fig. 1).

Subcloning of the herbicide resistance gene

The 1.7 Kb *PstI* insert was then subcloned into the high copy number streptomycete vector pIJ385 to generate plasmid pBG20. *S. lividans* strains which contained pBG20 were more than 500 times more resistant to Bialaphos. S. *lividans* growth is normally inhibited in minimal medium containing 1 µg/ml Bialaphos) growth of transformants containing pBG20 was not noticeably inhibited in a medium containing 500 µg/ml Bialaphos. The *PstI* fragment was also subcloned in either orientation into the *PstI* site of the plasmid pBR322, to produce plasmids pBG1 and pBG2, according to their orientation. A test on minimal M9 medium demonstrated that *E. coli* E8767 containing pBG1 or pBG2 was resistant to Bialaphos.

A ± 1.65 Kb *PstI- BamHI* fragment was subcloned from pBG1 into the plasmid pUC19 to produce the plasmid pBG39, and conferred Bialaphos resistance to *E. coli,* W3110, C600 and JM83.

Using an *in vitro* coupled transcription-translation system (ref. 5) from *S. lividans* extracts, the 1,65 Kb *PstI- BamHI* fragment in pBG39 was shown to direct the synthesis of a 22 Kd protein. In the following, this 1,65 Kb insert includes a fragment coding for a 22 Kd protein an will be called "sfr" gene.

Fine mapping and sequencing of the gene

A 625 bp *Sau*3A fragment was subcloned from pBG39 into pUC19 an still conferred Bialaphos resistance to a *E. coli* W3110 host. The resulting clones were pBG93 and pBG94, according to the orientation.

The orientation of the gene in the *Sau*3A fragment was indicated by experiments which have shown that Bialaphos resistance could be induced with IPTG from the pUC19 lac promoter in pBG93. In the presence of IPTG (0.5 mM) the resistance of pBG93/W3110 increased from 5 to 50 µg/ml on a M9 medium containing Bialaphos. The W3110 host devoid of pBG93, did not grow on M9 medium containing 5 µg/ml Bialaphos. These experiments demonstrated that the *Sau*3A fragment could be subcloned without loss of activity. They also provided for the proper orientation as shown in Fig. 2. The protein encoded by these clones was detected by using coupled transcription-translation systems derived from extracts of *S. lividans* (ref. 7). Depending on the orientation of the *Sau*3A fragment, translation products of different sizes were observed; 22 Kd for pBG94 and ± 28 Kd for pBG93. This indicated that the *Sau*3A fragment did not contain the entire resistance gene and that a fusion protein was formed which included a polypeptide sequence resulting from the translation of a pUC19 sequence.

In order to obtain large amounts of the protein, a 1.7 Kb *PstI* fragment from pBG1 was cloned into the high copy number Streptomycete replicon pIJ385. The obtained plasmid, pBG20, was used to transform *S. hygroscopicus*. Transformants which contained this plasmid had more than 5 times as much PPT acetylating activity and also had increased amounts of a 22 Kd protein on sodium dodecylsulfate gels (SDS gels). Furthermore, both the acetyl transferase and the 22 kd protein appeared when the production of Bialahos begun. The correlation of the *in vitro* data, kinetics of synthesis, and amplified expression associated with pBG20 transformants strongly implied that this 22 Kd band was the gene product.

The complete nucleotide sequence of the 625 bp *Sau*3A fragment was determined as well as of flanking sequences. Computer analysis revealed the presence of an open reading frame over the entire length of the *Sau*3A fragment.

Characterization of the sfr gene product

A series of experiments were performed to determine that the open reading frame of the "sfr" gene indeed encoded the Bialaphos resistance enzyme. To determine the 5' end of the resistance gene, the NH_2- terminal sequence of the enzyme was determined. As concerns more particularly the technique used to determine the said sequence, reference is made to the technique developed by J. VANDERKERCKHOVE, Eur, J. Bloc. *152*, p.9-19, 1985, and to French patent applications n° 85 14579 filed on October 1st, 1985 and n° 85 13046 filed on September 2nd, 1985, all of which are incorporated herein by reference.

This technique allows the immobilization on glass fibre sheets coated with the polyquaternary amine commercially available under the registered trademark POLYBRENE of proteins and of nucleic acids previously separated on a sodium dodecylsulfate containing polyacrylamide gel. The transfer is carried out essentially as for the protein blotting on nitrocellulose membranes (ref. 8). This allows the determination of amino-acid composition and partial sequence of the immobilized proteins. The portion of the sheet carrying the immobilized 22 kd protein produced by *S. hygroscopicus* pBG20 was cut out and the disc was mounted in the reaction chambre of a gas-phase sequenator to subject the glass-fibre bound 22 Kd protein to the Edman degradation procedure. The following amino-acid sequence was obtained:

Pro-Glu-Arg-Arg-Pro-Ala-Asp-Ile-Arg-Arg

This sequence matched an amino-acid sequence which was deduced from the open reading frame of the 625 bp *Sau*3A fragment. It corresponded to the stretch from codon 3 to codon 12.

Thus, the NH_2-terminus of the 22 Kd protein was upstream of this sequence. It was determined that translation of the actual protein was likely to be initiated 2 amino-acids earlier at a GTG initiation codon. GTG is often used as initiator codon in Streptomyces and translated as methionine. The protein translated from the GTG initiation codon would be 183 amino-acids long and would have a molecular weight of 20 550. This was in good agreement with the observed approximate molecular weight of 22 000.

Furthermore, the termination codon, TGA, was located just downstream of the Sau3A site. Cloning of the 625 bp *Sau*3A fragment in a *Bam*Hi site digested pUC19 did not result in the reconstruction of the termination codon. This explained the fusion proteins which were observed in the *in vitro* transcription-translation analysis.

Mechanism of PPT-resistance

Having defined a first phenotype and some of the physical characteristics of the resistance gene and its gene product, a series of experiments was then carried out to understand the mechanism by which it confers resistance. As described hereabove, PPT is the portion of Bialaphos which inhibits glutamine synthetase (GS) and that N-acetyl PPT is not an inhibitor. Using a standard assay (ref. 9), *S. hygroscopicus* ATCC 21 705 derivates were shown to contain a PPT acetyl transferase which was not found in *S. lividans*. The activity does not acetylate the Bialaphos tripeptide. *S. lividans* carrying the resistance gene cloned in pBG20 or pBG16 (a plasmid containing the 625 by Sau3A fragment cloned into another streptomycete vector, pIJ680) also contained the activity which could acetylate PPT but not Bialaphos. The PPT derived reaction product produced by extracts of pBG20/*S. lividans* was isolated in order to confirm that it was indeed acetyl-PPT. Analysis by mass spectroscopy showed that the molecular weight had increased relative to PPT by the equivalent of one acetyl group. It was thus concluded that the 625 bp Sau3A fragment contained sequences which code for PPT acetyl transferase.

The experimental conditions and reagents used in the techniques disclosed hereabove were as follows:

Preparation and composition of the mediums and buffers above used

1. *P medium:* 10.3 g of sucrose, 0.025 g of K_2SO_4, 0.203 g of $MgCl_2.6H_2O$ and 0.2 ml of a trace element solution are dissolved in 80 ml of distilled water and autoclaved. Then in order, 1 ml of KH_2PO_4 (0.5%), 10 ml of $CaCl_2$, $2H_2O$ (3.68%), and 10 ml of TES buffer (0.25 M), pH: 7.2) are added. Trace element solution (per litre): $ZnCl_2$, 40 mg; $FeCl_3$. $6H_2O$, 200 mg; $CuCl_2.2H_2O$, 10 mg; $MnCl_2.4H_2O$, 10 mg; $Na_2B_4O_7.10H_2O$, 10 mg; $(NH_4)_6Mo_7O_{24}.4H_2O$, 10 mg.

2: *R2YE:* 10.3 g of sucrose, 0.025 g of K_2SO_4, 1.012 g of $MgCl_2.6H_2O$, 1 g of glucose, 0.01 g of Difco casamino acids, and 2.2 g of Difco agar are dissived in 80 ml distilled water and autoclaved. 0.2 ml of trace element solution, 1 ml of KH_2PO_4 (0.5%), 8.02 ml of $CaCl_2.2H_2O$ (3.68%), 1.5 ml of L-proline (20%), 10 ml of TES buffer (0.25 M) (pH: 7.2), 0.5 ml of (1M) NaOH, 5 ml of yeast extract (10%) are sequentially added.

3. *TE:* 10 mM TRIS HCl, 1 mM EDTA, pH 8.0.

4. *YEME:* Difco yeast extract (0.3%), Difco peptone (0.5%), oxoid malt extract (0.3%), glucose (1%).

Transformation of S. lividans protoplasts

1. A culture composed of 25 ml YEME, 34% sucrose, 0.005 M $MgCl_2$, 0.5% glycine, in a 250 ml baffled flask, is centrifuged during 30 to 36 hours.

2. The pellet is suspended in 10.3% sucrose and centrifuged. This washing is repeated once.

3. The mycelium is suspended in 4 ml lysozyme solution (1 mg/ml in P medium with $CaCl_2$ and $MgCl_2$ concentrations reduced to 0.0025 M) and incubated at 30°C for 15 to 60 minutes.

4. The solution is mixed by pipetting three times in a 5 ml pipette and incubated for further 15 minutes.

5. P medium (5 ml) is added and mixed by pipetting as in step 4.

6. The solution is filtered through cotton wool and protoplasts are gently sedimented in a bench centrifuge at 800 × G during 7 minutes.

7. Protoplasts are suspended in 4 ml P medium and centrifuged again.

8. Step 7 is repeated and protoplasts are suspended in the drop of P medium left after pouring off the supernatant (for transformation).

9. DNA is added in less than 20 µl TE.

10.0.5 ml PEG 1 000 solution (2.5 g PEG dissolved in 7.5 ml of 2.5% sucrose, 0.0014 K_2SO_4, 0.1 M $CaCl_2$, 0.05 M TRIS-maleic acid, pH 8.0, plus trace elements) is immediately added and pipetted once to mix the components.

11. After 60 seconds, 5 ml of P medium are added and the protoplasts are sedimented by gentle centrifugation.

12. The pellet is suspended in P medium (1 ml).

13. 0.1 ml is plated out on R2YE plates (for transformation dry plates to 85% of their fresh weight e.g. in a laminar flow cabinet).

14. Incubation at 30°C.

A - Construction of a "sfr" gene cassette

A "sfr" gene cassette was constructed to allow subsequent cloning in plant expression vectors.

Isolation of a *FokI-BglII* fragment from the plasmid pBG39 containing a "sfr" gene fragment led to the loss of the first codons, including the initiation codon, and of the last codons, including the stop codon.

This fragment of the "sfr" gene could be reconstructed *in vitro* with synthetic oligonucleotides which encode appropriate amino-acids.

The complementary synthetic oligonucleotides were 5'-CATGAGCCCAGAAC and 3'-TCGGGTCTTGCTGC.

By using such synthetic oligonucleotides, the 5' end of the "sfr" gene could be reformed and the GTG initiation codon substituted by a codon well translated by plant cells, particularly an ATG codon.

The DNA fragment containing the oligonucleotides linked to the "sfr" gene was then inserted into an appropriate plasmid, which contained a determined nucleotide sequence thereafter designated by an "adapter" fragment.

This adapter fragment comprised:

a TGA termination codon which enabled the last condons of the "sfr" gene to be reformed;

appropriate restriction sites which enabled the insertion of the fragment of the nucleotide sequence comprising the "sfr" gene partially reformed with the synthetic oligonucleotides; this insertion resulted in the reconstruction of an intact "sfr" gene;

appropriate restriction sites for the isolation of the entire "sfr" gene.

The "sfr" gene was then inserted into another plasmid, which contained a suitable plant promoter sequence. The plant promoter sequence consisted of the cauliflower mosaic virus promoter sequence (p35S). Of course the invention is not limited to the use of this particular promoter. Other sequences could be chosen as promoters suitable in plants, for example the TR 1'-2' promoter region and the promoter fragment of a Rubisco small subunit gene from *Arabidopsis thaliana* hereinafter described.

1. Construction of the plasmid pLK56.2 (Fig. 3)

The construction of plasmid pLK56.2 aimed at obtaining a suitble adaptor including the following sequence of restriction sites: *Sma*I, *Bam*HI, *Nco*I, *Kpn*I, *Bgl*II, *Mlu*I, *Bam*HI, *Hind*III, and *Xba*I.

The starting plasmids used for this construction, pLK56, pJB64 and pLK33 were those disclosed by BOTTERMAN (ref. 11).

The DNA fragments hereafter described were isolated and separated from low melting point agarose (LGA).

The plasmid pLK56 was cleaved by the enzymes *Bam*HI and *Nde*I. A *Nco*I-*Nde*I fragment (referred to in the drawings by arc "a" in broken line) obtained from plasmid pJB64 was substituted in pLK56 for the *Bam*HI-*Nde*I fragment shown at "b". Ligation was possible after filling in the *Bam*HI and *Nco*I protruding ends with the DNA polymerase I of *E. coli* (Klenow's fragment).

Paticularly recircularization took place by means of a T4 DNA ligase. A new plamid pLK56.3 was obtained.

This plasmid was cleaved by the enzymes *Xba*I and *Pst*I.

The *Bam*Hi-*Pst*I fragment of pLK33 (c) (on Fig. 3) was substituted for the *Xba*I-*Pst*I fragment (d) of pLK56.3, after repairing of their respective ends by Klenow's fragment.

After recircularization by means of the T4 DNA ligase, the obtained plasmid pLK56.2 contained a nucleotide sequence which comprised the necessary restriction sites for the subsequent insertion of the "sfr" gene.

2. Construction of the plasmid pGSH150 (Fig. 4A)

Parallel with the last discussed construction, there was produced a plasmid containing a promoter sequence recognized by the polymerases of plant cells and including suitable restriction sites, downstream of said promoter sequence in the direction of transciption, which restriction sites are then intended to enable the accomodation of the "sfr" gene then obtainable from pLK56.2, under the control of said plant promoter.

Plasmid pGV825 is described in DEBLAERE et al. (ref. 10). *Plasmid* pJB63 is from BOTTERMAN (ref. 11).

pGV825 was linearized with *Pvu*II and recircularized by the T4 DNA ligase, resulting in the deletion of an internal

*Pvu*II fragment shown at (e), (plasmid pGV956).

pGV956 was then cleaved by *Bam*HI and *Bgl*II.

The *Bam*HI-*Bgl*II fragment (f) obtained from pJB63 was dephosphorylated with calf intestine phosphatas (CIP) and substituted for the *Bam*HI-*Bgl*II fragment of pGV956.

Plasmid pGV1500 was obtained after recircularization by means of T4 DNA ligase.

An *Eco*RI-*Hind*III fragment obtained from plasmid pGSH50 was purified. The latter plamid carried the dual TR 1'-2' promoter fragment described in VELTEN et al., (ref. 13). This fragment was inserted in pGV1500, digested with *Hpa*I and *Hind*III and yielded pGSH150.

The plasmid contains the promoter fragment in front of the 3' end of the T-DNA transcript 7 and a *Bam*HI and *Cla*I sites for cloning.

3. Construction of the plasmid pGSJ260 (Fig. 4B)

CP3 is a plasmid derived from pBR322 and which contains the 35S promoter region of cauliflower mosaic virus within a *Bam*HI fragment.

pGSH150 was cut by *Bam*HI and *Bgl*II.

The *Bam*HI-*Bgl*II fragment (h) of CP3, which contained the nucleotide sequence of p35S promoter, was substituted for the *Bam*HI-*Bgl*II fragment (i) in pGHSH150 to from plasmid pGSJ250. GSJ250 was then opened at its *Bgl*II restiction site.

A *Bam*HI fragment obtained from mGV2 (ref. 12) was inserted in pGSJ250 at the *Bgl*II site to form plasmid pGSJ260.

However prior to inserting the "sfr" gene obtainable from pLK56.2 into plasmid pGSJ260, it was still desirable to further modify the first in order to permit insertion in a more practical manner. Thus pLK56.2 was further modified as discussed below to yield pGSR1.

Starting from plasmid pGSJ260, two plasmid constructions for subsequent transformations of plant cells were made:

a first plasmid permitting the expression of the "sfr" gene in the cytoplasm of plant cells, and
a second plasmid so modified as to achieve transport of the Bialaphos-resistance enzymes to the chloroplasts of plant cells.

First case: plasmid enabling the expression of the "sfr" gene in the cytoplasm of plant cells Cloning of the sfr gene cassette in a plant expression vector (pGSR2) (Fig. 5)

On figure 5A, the nucleotide sequence of the adapter of pLK56.2 is shown. In particular, the locations of *Bam*HI, *Nco*I, *Ngl*II restriction sites are shown.

This adapter fragment was cleaved by the enzymes *Nco*I and *Bgl*II.

Figure 5B shows the *Fok*I-*Ngl*II fragment (j) obtained from pBG39. The locations of these two restriction sites are shown on figure 2.

Using synthetic oligonucleotides, the first codons of the "sfr" gene were reformed, particularly the 5' end of the gene in which a ATG initiation codon was substituted for the initial GTG codon.

This *Fok*I-*Bgl*II fragment completed with the synthetic oligonucleotides was then substituted in pLK56.2 for the *Nco*I-*Bgl*II fragment of the adapter. The 3' end of the gene was thus reformed too, after recircularization with T4 DNA ligase. The plasmid obtained, pGSR1, thus contained the entire "sfr" gene inserted in its adapter.

The plasmid pGSJ260 was then opened by *Bam*HI (Fig. 5C) and the *Bam*HI fragment obtained from pGSR1, which contained the entire "sfr" gene, was inserted into pGSJ260.

The obtained plasmid, pGSR2 (see Fig. 6A) contained a pBR322 replicon, a bacterial streptomycin resistance gene (SDM-SP-AD-transferase) and an engineered T-DNA consisting of:

the border fragments of the T-DNA;
a chimeric kanamycin gene which provided a dominant selectable marker in plant cells; and
a chimeric "sfr" gene.

The chimeric "sfr" gene consisting of:

the promoter region of the cauliflower mosaïc virus (p35S);
the "sfr" gene cassette as described in Fig. 5;
the 3' untranslated region, including the polyadenylation signal of T-DNA transcipt 7.
pGSR2 was introduced into *Agrobacterium tumefaciens* recipient C58ClRif^R (pGV2260) according to the procedure

described by DEBLAERE et al. (ref. 10).

This strain was used to introduce the chimeric "sfr" gene in *N. tabacum* SR_1 plants.

Two variant plasmids derived from pGSR2, namely pGSFR280 and pGSFR281, have been constructed. They differ in the untranslated sequence following the transcription initiation site. In pGSR2, this fragment consists of the following sequence:

GAGGACACGCGAAATCACCAGTCTCGGATCC*ATG*;

while is consists of:

GAGGACACGCTGAAATCACCAGTCTCTCTACAAATCGATCC*ATG*

in pGSR280 and of

GAGGACACGCTGAAATCACCAGTCTCTCTACAAATCG*ATG*

in pGSFR281, with an ATG codon being the initiation codon of the "sfr" gene. The "sfr" gene is also fused to the TR1'-2' promoter in the plasmid pGSH150 (Fig. 4A) yielding pGSFR160 and pGSFR161 (Fig. 6B). These plasmids contain slight differences in the pTR2 "sfr" gene configuration: the "sfr" gene is correctly fused to the endogenous gene 2'. ATG in pGSFR161 (for sequences see ref. 13), whereas 4 extra base pairs (ATCC) are present just ahead of the ATG codon in pGSFR160. Otherwise, plasmids pGSFR161 and pGSFR160 are completely identical.

All plasmids are introduced in *Agrobacterium* by cointegration in the acceptor plasmid pGV2260 yielding the respective plasmids pGSFR1280, pGSFR1281, pGSFR1160 and pGSFR1161.

Second case: construction of a plasmid containing the "sfr" gene downstream of a DNA sequence encoding a transit peptide and suitable for achieving subsequent translocation of the "sfr" gene expression product into plant-cellchloroplasts.

In another set of experiments, the nucleotide sequence which contained the "sfr" gene was fused to a DNA sequence encoding a transit peptide so as to enable its transport into chloroplasts.

A fragment of the "sfr" gene was isolated from the adapter fragment above described and fused to a transit peptide. With synthetic oligonucleotides, the entire "sfr" gene was reconstructed and fused to a transit peptide.

The plasmid (plasmid pATS3 mentioned below) which contained the nucleotide sequence encoding the transit peptide comprised also the promoter sequence thereof.

Construction of the plasmid pGSR4 which contains the "sfr" gene fused to a DNA sequence encoding transit peptide (Fig. 7)

Plasmid pLK57 is from BOTTERMAN, (ref. 11). Plasmid pATS3 is a pUC19 clone which contains a 2 Kb *Eco*RI genomic DNA fragment from *Arabidopsis thaliana* comprising the promoter region and the transit peptide nucleotide sequence of the gene encoding the small subunit of ribulose biphosphate carboxylase (ssu). The *A. thaliana* small subunit was isolated as a 1 500 bp *Eco*RI-*Sph*I fragment. The *Sph*I cleavage site exactly occurs at the site where the coding region of the mature ssu protein starts.

Plasmids pLK57 and pATS3 were opened with *Eco*RI and *Sph*I. After recircularization by means of the T4 DNA ligase, a recombinant plasmid pLKAB1 containing the sequence encoding the transit peptide (Tp) and its promoter region (Pssu) was obtained.

In order to correctly fuse the "sfr" gene at the cleavage site of the signal peptide, the N-terminal gene sequence was first modified. Since it was observed that N-terminal gene fusions with the "sfr" gene retain their enzymatic activity, the second codon (AGC) was modified to a GAC, yielding an *Nco*I site overlapping with the ATG initiator site. A new plasmid, pGSSFR2 was obtained. It only differs from pGSR1 (Fig. 5B), by that mutation. The *Nco*I-*Bam*HI fragment obtained from pGSFR2 was fused at the *Sph*I end of the transit peptide sequence. In parallel, the "sfr" gene fragment was fused correctly to the ATG initiator of the ssu gene (not shown in figures).

Introduction of the "sfr" gene into a different plant species

The Bialaphos-resistance induced in plants by the expression of chimeric genes, when the latter have been transformed with appropriate vectors containing said chimeric genes, has been demonstrated as follows. The recombinant plasmids containing the "sfr" gene were introduced separately by mobilization into *Agrobacterium* strain $C58C_1$ Rif^R (pGV2260) according to the procedure described by DEBLAERE and al., Nucl. Acid Res., 13, p. 1 477, 1985. Recom-

binant strains containing hybrid Ti plasmids were formed. These strains were used to inf ct and transform leaf discs of different plant species, according to a method essentially as described by HORSH and al., 1985, Science, vol. 227. Transformation procedure of these different plant species given by way of example, is described thereafter.

1. Leaf disc transformation of *Nicotiana tabacum*

Used Media are described thereafter:

A_1 MS Salt/2

+ 1% sucrose
0.8% agar
pH 5.7

A_{10} B5-medium

+ 250 mg/l NH_4NO_3
750 mg/l $CaCl_2$ $2H_2O$
0.5 g/l 2-(N-Morpholino)ethane-sulfonic acid (MES) pH 5.7
30 g/l sucrose

A_{11} B5-medium

+ 250 mg/l NH_4NO_3
0.5 g/l MES pH 5.7
2% glucose
0.8% agar
40 mg/l adenine
+ 1 mg/l 6-Benzylaminopurine (BAP)
0.1 mg/l Indole-3-acetic acid (1AA)
500 mg/l Claforan

A_{12} B5-Medium

+ 250 mg/l NH_4NO_3
0.5 g/l MES pH 5.7
2% glucose
0.8% agar
40 mg/l adenine

+ 1mg/l BAP
200 mg/l claforan

A_{13} MS-salt/2

+ 3% sucrose
0.5 MES g/l pH 5.7
0.7% agar
200 mg/l claforan

Bacterial medium = min A:

(Miller 1972) 60 mM
K_2HPO_4, $3H_2O$,
33 mM KH_2PO_4; 7.5 mM $(NH_4)_2SO4$
1.7 M trinatriumcitrat; 1 mM $MgSO_4$;
2 g/l glucose; 50 mg/l vitamine B1

Plant material:

Nicotiana tabacum cv. Petit Havana SR1

Plants are used 6 to 8 weeks after subculture on medium A_1

Infection:

midribs and edges are removed from leaves.

Remaining parts are cut into segments of about 0.25 cm^2 and are placed in the infection medium A_{10} (about 12 segments in a 9 cm Petri dish containing 10 ml A_{10}).

Segments are then infected with 25 µ ml per Petri dish of a late log culture of the *Agrobacterium* strain grown in min A medium.

Petri dish are incubated for 2 to 3 days at low light intensity.

After 2 to 3 days medium is removed and replaced by 20 ml of medium A_{10} containing 500 mg/l clarofan.

Selection and shoot induction

The leaf discs are placed on medium A_{11} containing a selective agent:

100 mg/l kanamycin and
10 to 100 mg/l phospinotricin.

Leaf discs are transferred to fresh medium weekly.

After 3 to 4 weeks regenerating calli arise. They are separated and placed on medium A_{12} with the same concentration of selective agent as used for the selection.

Rooting

After 2 to 3 weeks the calli are covered with shoots, which can be isolated and transferred to rooting medium A_{13} (without selection).

Rooting takes 1 to 2 weeks.

After a few more weeks, these plants are propogated on medium A_1.

2. Tuber disc infection of *Solanum tuberosum (potato)*

Used media are described thereafter:

C_1 B5-medium

+ 250 mg/l NH_4NO_3
300 mg/l $(CaCH_2PO_4)_2$
0.5 g/l MES pH 5.7
0.5 g/l polyvinylpyrrolidone (PVP)
40 g/l mannitol (=0.22M)
0.8% agar
40 mg/l adenine

C_2 B5-medium

+ 250 mg/l NH_4NO_3
400 mg/l glutamine
0.5 g/l MES pH 5.7
0.5 gl PVP
40 g/l mannitol
40 mg/l adenine
0.8% agar

+ 0.5 mg/l transzeatine
0.1 mg/l IAA
500 mg/l clarofan

C_5 MS salt/2

+3% sucrose
0.7% agar
pH 5.7

C_7 B5-medium

+ 250 mg/l NH_4NO_3
400 mg/l glutamine
0.5 g/l MES pH 5.7
0.5 g/l PVP
20 g/l mannitol
20 g/l glucose
40 mg/l adenine
0.6% agarose

+ 0.5 mg/l transzeatine
0.1 mg/l IAA
500 mg/l clarofan

C_8 B5-medium

+ 250 mg/l NH_4NO_3
400 mg/l glutamine
0.5 g/l MES pH 5.7
0.5 g/l PVP
20 g/l mannitol
20 g/l glucose
40 mg/l adenine
0.6% agarose

+ 200 mg/l clarofan
1 mg/l transzeatine

C_9 B5-medium

+ 250 mg/l NH_4NO_3
400 mg/l glutamine
0.5 g/l MES pH 5.7
0.5 g/l PVP
20 g/l mannitol
20 g/l glucose
40 mg/l adenine
0.6% agarose

+ 1 mg/l transzeatine
0.01 mg/l Gibberellic acid A_3 (GA_3)
100 mg/l clarofan

C_{11} MS salt/2

+ 6% sucrose
0.7% agar

Bacterial medium = min A;

(Miller 1972 60 mM $K_2HPO_4 \cdot 3H_2O$;
33 mM KH_2PO_4;
7.5 mM $(NH_4)_2SO_4$;
1.7 trinatriumcitrat; 1 mM
$MgSO_4$;
2 g/l glucose; 50 mg/l vitamine B1.

Plant material

Tubers of Solanum tuberosum c.v. Berolina c.v. Désirée

Infection

Potatoes are peeled and washed with water.
Then they are washed with concentrated commercial bleach for 20 minutes, and rinsed 3 to 5 times with sterile water.
The outer layer is removed (1 to 1.5 cm)
The central part is cut into discs of about 1 cm^2 and 2 to 3 mm thick.
Discs are placed on medium C_1 (4 pieces in a 9 cm Petri dish).
10 µl of a late log culture of an Agrobacterium strain grown in min A medium is applied on each disc.
Discs are incubated for 2 days at low light intensity.

Selection and shoot induction

Discs are dried on a filter paper and transferred to medium C_2 with 100 mg/l kanamycin.
After one month small calli are removed from the discs and transferred to medium C_7 containing 50 mg/l kanamycin.
After a few more weeks, the calli are transferred to medium C_8 containing 50 mg/l kanamycin.
If little shoots start to develop, the calli are transferred to elongation medium C_9 containing 50 mg/l Kanamycin.

Rooting

Elongated shoots are separated and transferred to rooting medium C_{11}.
Rooted shoots are propagated on medium C_5.

3. Leaf disc infection of *Lycopersicum esculentum* (tomato)

Used media are described thereafter

A_1 MS salt/2

+ 1% sucrose
0.8% agar
pH 5.7

B_1 B5-medium

+ 250 mg/l NH_4NO_3
0.5 g.l MES pH 5.7
0.5 g/l PVP
300 mg/l Ca $(H_2PO_4)_2$
2% glucose
40 mg/l adenine
40 g/l mannitol

B_2 B5-medium

+ 250 mg/l NH_4NO_3
0.5 g/l MES pH 5.7
0.5 g/l PVP
400 mg/l glutamine
2% glucose
0.6% agarose
40 mg/l adenine
40 g/l mannitol

+ 0.5 mg/l transzeatine
0.01 mg/l IAA
500 mg/l claforan

B_3 B5-medium

+ 250 mg/l NH_4NO_3
0.5 g/l MES pH 5.7
0.5 g/l PVP
400 mg/l glutamine
2% glucose
0.6% agarose
40 mg/l adenine
30 g/l mannitol

+ 0.5 mg/l transzeatine
0.01 mg/l IAA
500 mg/l clarofan

B_4 B5-medium

+ 250 mg/l NH_4NO_3
0.5 g/l MES pH 5.7
0.5 g/l PVP
400 mg/l glutamine
2% glucose
0.6% agarose
40 mg/l adenine
20 g/l mannitol

+ 0.5 mg/l transzeatine
0.01 mg/l IAA
500 mg/l clarofan

B_5 B5-medium

+ 250 mg/l NH_4NO_3
0.5 g/l MES pH 5.7
0.5 g/l PVP
400 mg/l glutamine
2% glucose
0.6% agarose
40 mg/l adenine
10 g/l mannitol

+ 0.5 mg/l transzeatine
0.01 mg/l IA
500 mg/l clarofan

Infection

Midrib is removed from the leaves.

Leaves are cut in segments of about 0.25 to 1 cm^2 (the edges of the leaves are not wounded, so that only maximum 3 sides of the leaf pieces is wounded).

Segments are placed in infection medium B_1 (upside down), about 10 segments in a 9 cm Petri dish.

Segments are then infected with 20 µl per Petri dish of a late log culture of the *Agrobacterium* strain grown in min A medium.

Petri dishes incubate for 2 days at low light intensity.

Medium is removed after 2 days and replaced by 20 ml of medium B_1 containing 500 mg/l clarofan.

Selection and shoot induction

The leaf discs are placed in medium B_2 + 50 or 100 mg/l kanamycin.

Each 5 days the osmotic pressure of the medium is lowered by decreasing the mannitol concentration, transfers are done consecutively in medium B_3, B_4, B_5 and B_6.

After one month calli with meristems are separated from the leaf discs and placed on medium B_7 with 50 or 100 mg/l kanamycin.

Once little shoots have formed, calli are transferred to elongation medium B_9 with 50 or 100 mg/l kanamycin.

Rooting

Elongated shoots are separated and transferred to medium B_8 for rooting.

Plants are propagated on medium A_1.

Greenhouse tests for herbicide resistance

Material and method

In this experiment, two herbicides comprising phosphinotricin as active ingredient, are used.

These compounds are those commercially available under the registered trademarks BASTA^R and MEIJI HERBIACE^R.

These products are diluted to 2% with tap water. Spraying is carried out on a square metre area from the four corners. Temperature of the greenhouse is about 22°C for tobacco and tomato, and above 10°C to 15°C for potato.

Results

Tobacco spraytest

a) *Nicotiana tabacum* cv. Petit Havana SR1 plants transformed with the chimeric "sfr" genes as present in pGSFR1161 or pGSFR1281, as well as unstransformed control plants (from 10 cm to 50 cm high) are treated with 20 l BASTA^R/ha. Control SR1 plants die after 6 days, while transformed plants are fully resistant to 20 l BASTA^R/ha and continue growing undistinguishable from untreated plants. No visible damage is detected, also if the treatment is repeated every two weeks. The treatment has no effect on subsequent flowering. The recommended dose of BASTA^R herbicide in agriculture is 2.5-7.5 l/ha.

b) A similar experiment is performed using 8 l/ha MEIJI HERBIACE^R. The transformed plants (the same as above) are fully resistant and continue growing undistinguishable from untreated plants. No visible damage is detectable.

Potato spraytest

Untransformed and transformed potato plants *(Solanum tuberosum* cv. Berolina) (20 cm high) with the chimeric "sfr" gene as present in pGSFR1161 or pGSFR1281 are treated with 20 l BASTA^R/ha. Control plants die after 6 days while the transformed plants do not show any visible damage. They grow undistiguishable from untreated plants.

tomato spraytest

Untransformed and transformed tomato plants (*lycopersicum esculentum* c.v. luculus) (25 cm high) with the chimeric "sfr" gene as present in pGSFR1161 and pGSFR1281 are treated with 20 l BASTA^R/ha. Control plants die after

six days while transformed plants are fully resistant. They do not show any visible damage and grow undistinguishable from untreated plants.

Growth control of phytopathogenic fungi with transformed plants.

In another set of experiments, potato plants expressing chimeric "sfr" genes are present in pGSFR1161 or pGSFR1281 are grown in a greenhouse compartment at 20°C under high humidity. Plants are inoculated by spraying 1 l of a suspension of 10^6 *Phytophtora infestans* spores per ml. Plants grow in growth chambers (20°C, 95% humidity, 4,000 lux) until fungal disease symptoms are visible (one week). One set of the plants are at the moment sprayed with Bialaphos at a dose of 8 l/ha. Two weeks later, untreated plants are completely ingested by the fungus. The growth of the fungus is stopped on the Bialaphos treated plants and no further disease symptoms evolve. The plants are effectively protected by the fungicide action of Bialaphos.

Transmission of the PPT resistance through seeds

Transformed tobacco plants expressing the chimeric "sfr" gene present in pGSFR1161 and pGSFR1281 are brought to flowering in the greenhouse. They show a normal fertility.

About 500 F1 seeds of each plant are sown in soil, F1 designating seeds of the first generation, i.e. directly issued from the originally transformed plants. When seedlings are 2-3 cm high, they are sprayed with 8 l BASTRA^R/ha. 7 days later, healthy and damaged plants can be distinguished in a ratio of approximately 3 to 1. This shows that PPT resistance is inherited as a dominant marker encoded by a single locus.

10 resistant F1 seedlings are grown to maturity and seeds are harvested. F2 seedlings are grown as described above and tested for PPT-resistance by spraying BASTA^R at a dose of 8 l/ha. Some of the F1 plants produce F2 seedlings which are all PPT-resistant showing that these plants are homozygous for the resistance gene. The invention also concerns plant cells and plants non-essentially-biologically-transformed with a GS inhibitor-inactivating-gene according to the invention.

In a preferred embodiment of the invention, plant cells and plants are non-biologically-transformed with the "sfr" gene hereabove described.

Such plant cells and plants possess, stably integrated in their genome, a non-variety-specific character which render them able to produce detectable amounts of phosphinotricin-acetyl transferase.

This character confers to the transformed plant cells and plants a non-variety-specific enzymatic activity capable of inactivating or neutralizing GS inhibitors like Bialaphos and PPT.

Accordingly, plant cells and plants transformed according to the invention are rendered resistant against the herbicidal effects of Bialaphos and related compounds.

Since Bialaphos was first described as a fungicide, transformed plants can also be protected against fungal diseases by spraying with the compound several times.

In a preferred embodiment, Bialaphos or related compounds is applied several times, particularly at time intervals of about 20 to 100 days.

The invention also concerns a new process for selectively protecting a plant species against fungal diseases and selectively destroying weeds in a field comprising the steps of treating a field with an herbicide, wherein the plant species contain in their genome a DNA fragment encoding a protein having an enzymatic activity capable of neutralizing or inactivating GS inhibitors and wherein the used herbicide comprises as active ingredient a GS inhibitor.

It comes without saying that the process according to the invention can be employed with the same efficiency, either only to destroy weeds in a field, if plants are not infected with fungi, or only to stop the development of fungi if the latter appears after destruction of weeds.

In a preferred embodiment of the process according to the invention, plant species are transformed with a DNA fragment comprising the "sfr" gene as described hereabove, and the used herbicide is PPT or a related compound.

Accordingly, a solution of PPT or related compound is applied over the field, for example by spraying, several times after emergence of the plant species to be cultivated, until early and late germinating weeds are destroyed.

It is quite evident that before emergence of plant species to be cultivated, the field can be treated with an herbicidal composition to destroy weeds.

On the same hand, fields can be treated even before the plant species to be cultivated are sowed.

Before emergence of the desired plant species, fields can be treated with any available herbicide, includign Bialaphos-type herbicides.

After emergence of the desired plant species, Bialaphos or related compound is applied several times.

In a preferred embodiment, the herbicide is applied at time intervals of about from 20 to 100 days.

Since plants to be cultivated are transformed in such a way as to resist to the herbicidal effects of Bialaphos-type herbicides, fields can be treated even after emergence of the cultivated plants.

This is particularly useful to totally destroy early and late germinating weeds, without any effect on the plants to be produced.

Preferably, Bialaphos or related compound is applied at a dose ranging from about 0.4 to about 1.6 kg/ ha, and diluted in a liquid carrier at a concentration such as to enable its application to the field at a rate ranging from about 2 to about 8 l/ha.

There follows examples, given by way of illustration, of some embodiments of the process with different plants species.

Sugarbeets

The North European sugarbeet is planted from March 15 up to April 15, depending upon the weather condition and more precisely on the precipitation and average temperature. The weed problems are more or less the same in each country and can cause difficulties until the crop closes its canopy around mid-July.

Weed problems can be separated in three situations:

early germination of the grassy weeds,
early geminating broadleaved weeds,
late germinating broadleaved weeds.

Up to now, pre-emergence herbicides have been successfully used. Such compounds are for example those commercially available under the registered trademarks: PYRAMIN®, GOLTIX® and VENZAR®. However, the susceptibility to dry weather conditions of these products as well as the lack of residual activity to control late germinating weeds have led the farmer to use post-emergence products in addition to pre-emergence ones.

Table (I) thereafter indicates the active ingredients contained in the herbicidal compositions cited in the following examples.

TABLE (I)

Commercial Name	Active Ingredient	Formulation
AVADEX®	Diallate	EC 400 g/l
AVADEX BW®	Triallate	EC 400 g/l
GOLTIX®	Metamitron	WP 70%
RONEET®	Cycloate	EC 718 g/l
TRAMAT®	Ethofumerate	EC 200 g/l
FERVINAL®	Alloxydime-sodium	SP 75%
BASTA®	Phosphinotricin	200 g/l
PYRAMIN FL®	Chloridazon	SC 430 g/l

According to the invention, post-emergence herbicides consist of Bialaphos or related compounds, which offer a good level of growth control of annual grasses (*Bromus, Avena* supp., *Alopecurus,* POA) and broadleaves (*Galium, Polygonum, Senecio, Solanum, Mercurialis*).

Post-emergence herbicides can be applied at different moments of the growth of sugarbeet; at a cotyledon level, two-leave level or at a four-leave level.

Table (II) thereafter represents possible systems of field-treatment, given by way of example.

In those examples, the post-emergence herbicide of the class of Bialaphos used is BASTA^R, in combination with different pre-emergence herbicides. Concentrations are indicated in 1/ha or kg/ha.

TABLE (II)

Possible weed control systems in sugarbeets, based on the use of BASTA^R, providing beets are made resistant against the laiter chemical (in lt or kg/ha).								
	Pre-sowing	Pre-emergence	Colyledons		Two-leaves		Four leaves	
1.	AVADEX® 3.5 lt	-	BASTA® 3 lt		BASTA®/tramat 3 lt	1.5 lt	-	
2.	AVADEX® 3.5 lt	GOLTIX® 4 kg	-		-		-	

TABLE (II) (continued)

	Pre-sowing	Pre-emergence	Colyledons		Two-leaves		Four leaves	
3.	RONEET® 4 lt	GOLTIX® 5 kg	-		-		-	
4.	RONEET® 4 lt	GOLTIX® 2.5 kg	- -		BASTA® 3 lt		- -	
5.	TRAMAT® 5 lt	-	-		BASTA® 3 lt		BASTA®/GOLTIX® 2 lt	2kg
6.	-	GOLTIX® 2.5 kg	-		BASTA® 3 lt		-	
7.	-	-	BASTA®/tramat 3 lt	1.7 lt	- -		BASTA®/GOLTIX® 3 lt	2 kg
8.	PYRAMIN® 6 lt	-	BASTA® 3 lt		Venzar 1 kg		-	
9.	-	-	BASTA® 3 lt		BASTA®/GOLTIX® 3 lt	2 kg	-	
10.	DIALLATE® 3.5 lt	PYRAMIN® 6 lt			BASTA®/Metamitron 3 lt	1 kg	-	

Potatoes

Potatoes are grown in Europe on about 8.10^6 Ha. The major products used for weed control are Linuron/monolinuron or the compound commercially available under the denomination METRABUZIN.

The products perform well against most weedspecies.

However, weeds such as *Galium* and *Solanum* plus late germinating *Chenopodium* and *Polygonum* are not always effectively controlled, while control of the annual grasses is also sometime erratic.

Once again, late germinating broadleaved weeds are only controllable by post-emergence applications of herbicides such as BASTA^R.

Table (III) thereafter represents some examples given by way of example of field-treatment in the case of potatoes.

TABLE (III)

Weeds control systems in potatoes, based on the use of BASTA®, providing potatoes are rendered resistant to BASTA®. Linuron + monolinuron (375 g + 375 g/ha) prior to emergence	
BASTA^R	3-4 lt/ha after emergence (5-15 cm)
BASTA^R/flazifop-butyl	3-4 lt/ha + 2 lt/ha after emergence (5-15 cm)
Lunuron	WP 50% (AFALON^R)
Monolinuron	WP 47.5% (ARESSIN^R)
fluazifop-butyl	EL 250 g/l (FUSILADE^R)

The strains pGSJ260 and pBG39 used hereabove have been deposited on December 12th, 1985, at the "German Collection of Micro-organisms" (Deutsche Sammlung von Mikroorganismen) at Gùttingen, Germany. They received the deposition numbers DSM 3 606 and DSM 3 607 respectively.

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Claims

1. Process for controlling the action in plant cells and plants comprising such cells of a glutamine synthetase inhibitor when the former are contacted with the latter, which comprises causing the stable integration in the genomic DNA of said plant cells of a heterologous DNA including a promoter recognized by polymerases of said plant cells and a foreign nucleotide sequence capable of being expressed in the form of a protein in said plant cells and plants, under the control of said promoter, and wherein said protein has an enzymatic activity capable of causing inactivation or neutralization of said glutamine synthetase inhibitor.

2. Process according to claim 1, wherein the heterologous DNA fragment comprises a foreign nucleotide sequence coding for a polypeptide having an acetyl transferase activity, particularly PPT ucetyltransferase activity, with respect to said glutamine synthetase inhibitor.

3. Process according to claims 1 or 2, wherein the foreign nucleotide sequence is derived from the genome of an antibiotic-producing *Streptomyces* strain or is a nucleotide sequence encoding the same activity.

4. Process according to any of the claims 1 to 3, wherein the heterologous DNA comprises a foreign nucleotide sequence coding for a protein having the following sequence:

X SER PRO GLU

183
ARG ARG PRO ALA ASP ILE ARG ARG ALA THR GLU ALA ASP MET PRO
228
ALA VAL CYS THR ILE VAL ASN HIS TYR ILE GLU THR SER THR VAL
273
ASN PHE ARG THR GLU PRO GLN GLU PRO GLN GLU TRP THR ASP ASP
318
LEU VAL ARG LEU ARG GLU ARG TYR PRO TRP LEU VAL ALA GLU VAL
363
ASP GLY GLU VAL ALA GLY ILE ALA TYR ALA GLY PRO TRP LYS ALA
408
ARG ASN ALA TYR ASP TRP THR ALA GLU SER THR VAL TYR VAL SER
453
PRO ARG HIS GLN ARG THR GLY LEU GLY SER THR LEU TYR THR HIS
498
LEU LEU LYS SER LEU GLU ALA GLN GLY PHE LYS SER VAL VAL ALA
543
VAL ILE GLY LEU PRO ASN ASP PRO SER VAL ARG MET HIS GLU ALA
588
LEU GLY TYR ALA PRO ARG GLY MET LEU ARG ALA ALA GLY PHE LYS
633
HIS GLY ASN TRP HIS ASP VAL GLY PHE TRP GLN LEU ASP PHE SER
678
LEU PRO VAL PRO PRO ARG PRO VAL LEU PRO VAL THR GLU ILE
723

in which X represents MET or VAL or for part of said protein, wherein said part is of sufficient length to possess the enzymatic activity of the full protein and, when said DNA fragment is expressed in plant cells, to protect the latter against the herbicidal activity of said glutamine synthetase inhibitor.

5. Process according to any of the claims 1 to 4, wherein the heterologous DNA fragment comprises the following nucleotide sequence:

```
                                                        GTG AGC CCA GAA
      183
CGA CGC CCG GCC GAC ATC CGC CGT GCC ACC GAG GCG GAC ATG CCG
      228
GCG GTC TGC ACC ATC GTC AAC CAC TAC ATC GAG ACA AGC ACG GTC
      273
AAC TTC CGT ACC GAG CCG CAG GAA CCG CAG GAG TGG ACG GAC GAC
      318
CTC GTC CGT CTG CGG GAG CGC TAT CCC TGG CTC GTC GCC GAG GTG
      363
GAC GGC GAG GTC GCC GGC ATC GCC TAC GCG GGC CCC TGG AAG CCA
      409
CGC AAC GCC TAC GAC TGG ACG GCC GAG TCG ACC GTG TAC GTC TCC
      453
CCC CGC CAC CAG CGG ACG GGA CTG GGC TCC ACG CTC TAC ACC CAC
      498
CTG CTG AAG TCC CTG GAG GCA CAG GGC TTC AAG AGC GTG GTC GCT
      543
GTC ATC GGG CTG CCC AAC GAC CCG AGC GTG CGC ATG CAC GAG GCG
      588
CTC GGA TAT GCC CCC CGC GGC ATG CTG CGG GCG GCC GGC TTC AAG
      633
CAC GGG AAC TGG CAT GAC GTG GGT TTC TGG CAG CTG GAC TTC AGC
      678
CTG CCG GTA CCG CCC CGT CCG GTC CTG CCC GTC ACC GAG ATC
      723
```

or the corresponding part thereof.

6. Process according to any of claims 1 to 4, wherein the heterologous DNA fragment comprises the nucleotide sequence of claim 5 having the initiation codon ATG substituted for the initiation codon GTG.

7. A process for producing a plant or reproduction material of said plant including a heterologous genetic material stably integrated therein and capable of being expressed in said plants or reproduction material in the form of a protein capable of inactivating of neutralizing the activity of a glutamine synthetase inhibitor, which process comprises transforming cells or tissue of said plants with a DNA recombinant containing a heterologous DNA including a foreign nucleotide sequence encoding said protein as well as the regulatory elements selected among those which are capable of causing the stable integration of said heterologous DNA in said plant cells or tissue and of enabling the expression of said foreign nucleotide sequence in said plant cells or plant tissue, regenerating plants or reproduction material of said plants or both from the plants cells or tissue transformed with said heterologous DNA and, optionally, biologically replicating said last mentioned plants or reproduction material or both.

8. The process according to claim 7, wherein starting cells are transformed with a recombinant DNA which contains the heterologous DNA as defined in any one of claims 2 to 6.

9. The process according to claim 8, wherein the recombinant DNA is a vector suitable for the transformation of the cells of said plant.

10. The process of any of claim 8 or 9, which confers resistance against herbicidal effects of Bialaphos, PPT or related derivatives to the transformed plant cells.

11. The process of any one of claims 7 to 10, wherein said heterologous DNA fragment comprises a nucleotide sequence encoding a transit peptide intercalated between said plant promoter region and said foreign nucleotide sequence coding for said glutamine synthetase inhibitor.

12. The process of claim 11, wherein the transit peptide is selected from ribulose-1,5 biophosphate carboxylase and chlorophyll a/b binding proteins.

13. The process of any of claims 8 to 12, wherein said vector is a Ti plasmid containing said heterologous DNA fragment.

14. Plant cells, non biologically transformed, which possess a heterologous DNA stably integrated in their genome, said heterologous DNA contianing a foreign nucleotide sequence encoding a protein having a non-variety specific enzymatic activity capable of neutralizing or inactivating a glutamine synthetase inhibitor under the control of a promotor recognized by the polymerases of said plant cells.

15. Plant cells according to claim 14, which can be regenerated into a plant capable of producing seeds.

16. Plant cells according to claim 14 or 15, which are transformed by the process of any of claims 7 to 13.

17. Plant cells according to any of claims 14 to 18, which produce detectable amounts of phosphinotricin acetyl transferase.

18. Process for selectively protecting the culture of a plant species and selectively destroying weeds which comprises the steps of treating the field with a herbicide consisting of a glutamine synthetase inhihitor, wherein the cells of the plant species contain in their genome a foreign nucleotide sequence encoding a protein having an enzymatic activity capable of neutralizing or inactivating said glutamine synthetase inhibitor under the control of a promoter recognized by the polymerases of the cells of said plant.

19. Process according to claim 18, wherein the plant species contain a heterologous DNA fragment as defined in any one of claims 1 to 6.

20. Process according to claim 18 or 19, wherein the plant species is transformed according to the process of any one of claims 7 to 13.

21. Process according tn any one of claims 18 to 20, wherein a solution of the glutamine synthetase inhibitor is applied on the field after emergence of the cultivated plant species, several times, particularly at time intervals of about 20 to 100 days, until early and late germinatting weeds are destroyed.

22. Process according to any one of claims 18 to 21, wherein the glutamine synthetase inhibitor is selected from a group which comprises Bialaphos, phosphinotricin or related compounds.

23. Process for selectively protecting a plant species in a filed against fungal diseases comprising the steps of treating said field with a herbicide consisting of a glutamine synthetase inhibitor, wherein the plant species contains in the genome of its cells a heterologous DNA including a promoter recognized by the polymerases of said cell and a foreigh nucleotide sequence encoding a protein having an enzymatic activity capable of neutralizing or inactivating said glutamine synthetase inhibitor under the control of said promoter.

24. Process according to claim 23, wherein the plant species contains a heterologous DNA as defined in any of claims 2 to 6.

25. Process according to claim 23 or 24, wherein the plant species is transformed according to the process of any one of claims 7 to 13.

26. Process according to any one of claims 23 to 25, wherein a solution of the glutamine synthetase inhibitor is applied on the filed after emergence of cultivated plant species, several times, particularly at time intervals of about 20 to 100 days until the fungi are destroyed.

27. Process according to any one of claims 23 to 26 wherein the glutamine synthetase inhibitor is selected from a group which comprises Bialaphos, phosphinotricin and related compounds.

28. Process according to any one of claims 18 to 27, wherein said Bialaphos, PPT or related compound is applied at a dose ranging form about 0.4 to about 1.6 kg/ha.

29. Process according to claim 28, wherein said Bialaphos, PPT or related compound is diluted in a liquid carrier at a concentration such as to enable its application to the filed at a rite ranging from about 2 l/h to about 8 l/ha.

30. Process according to claim 28 or 29 which is selectively applied to the protection of plant species selected from sugar-beet, rice, potato, tomato, maize, tobacco.

31. Vector for the transformation of plant cells which contains a heterologous DNA containing a promoter recognized by polymerases of cells of said plant and a foreign nucleotide sequence which codes for a polypeptide having an enzymatic activity capable of causing inactivation or neutralization of a glutamine synthetase inhibitor under the control of said promoter.

32. Vector according to claim 31, which contains a foreign nucleotide sequence coding for polypeptide having acetyl transferase activity, particularly PPT acetyltransferase activity.

33. Vector according to claim 32, which comprises the regulation elements required for the stable integration of said foreign nucleotide sequence in the genomic DNA of said plant cells and plants

34. Vector according to any of claims 31 to 33, which contains a DNA fragment belonging to the genome of an antibiotic-producting-*Streptomyces* strain.

35. Vector according to any of claims 31 to 34, wherein the foreign nucleotide sequence codes for a protein having the following sequence:

```
                                                  X SER PRO GLU
        183
ARG ARG PRO ALA ASP ILE ARG ARG ALA THR GLU ALA ASP MET PRO
        228
ALA VAL CYS THR ILE VAL ASN HIS TYR ILE GLU THR SER THR VAL
        273
ASN PHE ARG THR GLU PRO GLN GLU PRO GLN GLU TRP THR ASP ASP
        318
LEU VAL ARG LEU ARG GLU ARG TYR PRO TRP LEU VAL ALA GLU VAL
        363
ASP GLY GLU VAL ALA GLY ILE ALA TYR ALA GLY PRO TRP LYS ALA
        408
ARG ASN ALA TYR ASP TRP THR ALA GLU SER THR VAL TYR VAL SER
        453
PRO ARG HIS GLN ARG THR GLY LEU GLY SER THR LEU TYR THR HIS
        498
```

```
LEU LEU LYS SER LEU GLU ALA GLN GLY PHE LYS SER VAL VAL ALA
    543
VAL ILE GLY LEU PRO ASN ASP PRO SER VAL ARG MET HIS GLU ALA
    588
LEU GLY TYR ALA PRO ARG GLY MET LEU ARG ALA ALA GLY PHE LYS
    633
HIS GLY ASN TRP HIS ASP VAL GLY PHE TRP GLN LEU ASP PHE SER
    678
LEU PRO VAL PRO PRO ARG PRO VAL LEU PRO VAL THR GLU ILE
    723
```

in which X represents MET or VAI, or for part of said protein wherein said part is of sufficient length to possess said enzymatic activity and, when said DNA fragment is expressed in plant cells, to protect the latter against the herbicidal activity of a glutamine synthetase inhibitor.

36. Vector according to claim 35, which comprise the following nucleotide sequence:

```
                                                    Y AGC CCA GAA
    183
CGA CGC CCG GCC GAC ATC CGC CGT GCC ACC GAG GCG GAC ATG CCG
    228
GCG GTC TGC ACC ATC GTC AAC CAC TAC ATC GAG ACA AGC ACG GTC
    273
AAC TTC CGT ACC GAG CCG CAG GAA CCG CAG GAG TGG ACG GAC GAC
    318
CTC GTC CGT CTG CGG GAG CGC TAT CCC TGG CTC GTC GCC GAG GTG
    363
GAC GGC GAG GTC GCC GGC ATC GCC TAC GCG GGC CCC TGG AAG GCA
    408
CGC AAC GCC TAC GAC TGG ACG GCC GAG TCG ACC GTG TAC GTC TCC
    453
CCC CGC CAC CAG CGG ACG GGA CTG GGC TCC ACG CTC TAC ACC CAC
    498
CTG CTG AAG TCC CTG GAG GCA CAG GGC TTC AAG AGC GTG GTC GCT
    543
CTC ATC CGG CTG CCC AAC GAC CCG AGC GTG CGC ATG CAC GAG GCG
    588
CTC GGA TAT GCC CCC CGC GGC ATC CTG CGG GCG GCC GGC TTC AAG
    633
```

CAC GGG AAC TGG CAT GAC GTG GGT TTC TGG CAG CTG GAC TTC AGC
678
CTG CCG GTA CCG CCC CGT CCG GTC CTG CCC GTC.ACC GAG ATC
723

wherein Y is either ATG or GTG.

37. Vector according to any of claims 31 to 36, wherein said heterologous DNA comprises a necleotide sequence encoding a transit peptide intercalated between said plant promoter region and said foreign nucleotide sequence coding for said glutamine synthetase inhibitor.

38. Vector according to claim 37, which is a Ti plasmid containing said heterologous DNA.

Patentansprüche

1. Verfahren zur Steuerung der Wirkung in Pflanzenzellen und Pflanzen, umfassend solche Zellen, eines Glutamin-Synthetase-Inhibitors, wenn erstere mit letzterem in Kontakt gebracht werden, wobei das Verfahren das Verursachen der stabilen Integration in die genomische DNA dieser Pflanzenzellen einer heterologen DNA umfaßt, die einen Promoter, der durch Polymerasen dieser Pflanzenzellen erkannt wird und eine fremde Nukleotid-Sequenz einschließt, die in Form eines Proteins in diesen Pflanzenzellen und Pflanzen unter der Kontrolle dieses Promotors exprimiert werden kann, und worin dieses Protein eine enzymatische Aktivität aufweist, die die Inaktivierung oder Neutralisierung dieses Glutamin-Synthetase-Inhibitors verursachen kann.

2. Verfahren nach Anspruch 1, worin das heterologe DNA-Fragment eine fremde Nukleotid-Sequenz umfaßt, die für ein Polypeptid mit einer Acetyltransferase-Aktivität, insbesondere PPT-Acetyltransferase-Aktivität, bezüglich des Glutamin-Synthetase-Inhibitors, kodiert.

3. Verfahren nach Anspruch 1 oder 2, worin die fremde Nukleotid-Sequenz aus dem Genom eines Antibiotikumproduzierenden Streptomyces-Stammes abgeleitet ist oder eine Nukleotid-Sequenz, die für dieselbe Aktivität kodiert; ist.

4. Verfahren nach einem der Ansprüche 1 bis 3, worin die heterologe DNA eine fremde Nukleotid-Sequenz umfaßt, die für ein Protein mit der folgenden Sequenz:

X SER PRO GLU

183
ARG ARG PRO ALA ASP ILE ARG ARG ALA THR GLU ALA ASP MET PRO
228
ALA VAL CYS THR ILE VAL ASN HIS TYR ILE GLU THR SER THR VAL
273
ASN PHE ARG THR GLU PRO GLN GLU PRO GLN GLU TRP THR ASP ASP
318
LEU VAL ARG LEU ARG GLU ARG TYR PRO TRP LEU VAL ALA GLU VAL
363
ASP GLY GLU VAL ALA GLY ILE ALA TYR ALA GLY PRO TRP LYS ALA
408
ARG ASN ALA TYR ASP TRP THR ALA GLU SER THR VAL TYR VAL SER
453
PRO ARG HIS GLN ARG THR GLY LEU GLY SER THR LEU TYR THR HIS
498
LEU LEU LYS SER LEU GLU ALA GLN GLY PHE LYS SER VAL VAL ALA
543
VAL ILE GLY LEU PRO ASN ASP PRO SER VAL ARG MET HIS GLU ALA
588
LEU GLY TYR ALA PRO ARG GLY MET LEU ARG ALA ALA GLY PHE LYS
633
HIS GLY ASN TRP HIS ASP VAL GLY PHE TRP GLN LEU ASP PHE SER
678
LEU PRO VAL PRO PRO ARG PRO VAL LEU PRO VAL THR GLU ILE
723

in welcher X MET oder VAL darstellt oder für einen Teil des Proteins kodiert, worin dieser Teil genügend lang ist, um die enzymatische Aktivität des gesamten Proteins zu besitzen, und wenn das DNA-Fragment in Pflanzenzellen exprimiert wird, um letztere gegen die Herbizid-Aktivität des Glutamin-Synthetase-Inhibitors zu schützen.

5. Verfahren nach einem der Ansprüche 1 bis 4, worin das heterologe DNA-Fragment die folgende Nukleotid-Sequenz:

```
                                                    GTG AGC CCA GAA
        183
CGA CGC CCG GCC GAC ATC CGC CGT GCC ACC GAG GCG GAC ATG CCG
        228
GCG GTC TGC ACC ATC GTC AAC CAC TAC ATC GAG ACA AGC ACG GTC
        273
AAC TTC CGT ACC GAG CCG CAG GAA CCG CAG GAG TGG ACG GAC GAC
        318
CTC GTC CGT CTG CGG GAG CGC TAT CCC TGG CTC GTC GCC GAG GTG
        363
GAC GGC GAG GTC GCC GGC ATC GCC TAC GCG GGC CCC TGG AAG GCA
        409
CGC AAC GCC TAC GAC TGG ACG GCC GAG TCG ACC GTG TAC GTC TCC
        453
CCC CGC CAC CAG CGG ACG GGA CTG GGC TCC ACG CTC TAC ACC CAC
        498
CTG CTG AAG TCC CTG GAG GCA CAG GGC TTC AAG AGC GTG GTC GCT
        543
GTC ATC GGG CTG CCC AAC GAC CCG AGC GTG CGC ATG CAC GAG GCG
        588
CTC GGA TAT GCC CCC CGC GGC ATG CTG CGG GCG GCC GGC TTC AAG
        633
CAC GGG AAC TGG CAT GAC GTG GGT TTC TGG CAG CTG GAC TTC AGC
        678
CTG CCG GTA CCG CCC CGT CCG GTC CTG CCC GTC ACC GAG ATC
        723
```

oder den entsprechenden Teil davon umfaßt.

6. Verfahren nach einem der Ansprüche 1 bis 4, worin das heterologe DNA-Fragment die Nukleotid-Sequenz von Anspruch 5 umfaßt, bei der das Initiationskodon GTG durch das Initiationskodon ATG ersetzt ist.

7. Verfahren zur Herstellung einer Pflanze oder von Reproduktionsmaterial dieser Pflanze, einschließend ein heterologes genetisches Material das darin fest integriert ist und in diesen Pflanzen oder im Reproduktionsmaterial in Form eines Proteins exprimiert werden kann, das die Aktivität eines Glutamin-Synthetase-Inhibitors inaktivieren oder neutralisieren kann, wobei das Verfahren

   das Transformieren von Zellen oder Gewebe dieser Pflanzen mit einer rekombinanten DNA, die eine heterologe DNA einschließlich einer fremden Nukleotidsequenz enthält,
   die für das Protein kodiert, eben so wie Regulatorelemente, ausgewählt aus denen, die die stabile Integration der heterologen DNA in diese Pflanzenzellen oder das Gewebe verursachen können und die Expression der fremden Nukleotidsequenz in diesen Pflanzenzellen oder dem Pflanzengewebe ermöglichen und

das Regenerieren von Planzen oder Reproduktionsmaterial dieser Pflanzen oder von beidem aus den Pflanzenzellen oder dem Gewebe, die mit der heterologen DNA transformiert wurden, und, gegebenenfalls, biologisches Replizieren der letztgenannten Pflanzen oder des Reproduktionsmaterials oder von beiden umfaßt.

8. Verfahren nach Anspruch 7, worin die Ausgangszellen mit einer rekombinanten DNA transformiert werden, die die heterologe DNA wie in einem der Ansprüche 2 bis 6 definiert, enthält.

9. Verfahren nach Anspruch 8, worin die rekombinante DNA ein Vektor ist, der zur Transformation der Zellen dieser Pflanze geeignet ist.

10. Verfahren nach Anspruch 8 oder 9, das den transformierten Pflanzenzellen Resistenz gegen herbizide Effekte von Bialaphos, PPT oder verwandten Derivaten liefert.

11. Verfahren nach einem der Ansprüche 7 bis 10, worin das heterologe DNA-Fragment eine Nukleotidsequenz umfaßt, die ein Transitpeptid kodiert, das zwischen den Pflanzenpromotorbereich und die für den Glutamin-Synthetase-Inhibitor kodierende. fremde Nukleotidsequenz, eingeschoben ist.

12. Verfahren nach Anspruch 11, worin das Transitpeptid ausgewählt ist unter Ribulose-1,5-biphosphat-Carboxylase und Chlorophyll a/b bindenden Proteinen.

13. Verfahren nach einem der Ansprüche 8 bis 12, worin der Vektor ein Ti-Plasmid ist, das das heterologe DNA-Fragment enthält.

14. Nicht-biologisch transformierte Pflanzenzellen, die eine heterologe DNA stabil in ihrem Genom integriert besitzen, wobei die heterologe DNA eine fremde Nukleotidsequenz enthält, die für ein Protein mit einer nicht-Sorten-spezifischen enzymatischen Aktivität kodiert, die einen Glutamin-Synthetase-Inhibitor neutralisieren oder inaktivieren kann, unter der Kontrolle eines durch die Polymerasen dieser Pflanzenzellen erkannten Promotors.

15. Pflanzenzellen nach Anspruch 14, die zu einer Samenproduzierenden Pflanze regeneriert werden können.

16. Pflanzenzellen nach Anspruch 14 oder 15, die durch das Verfahren nach einem der Ansprüche 7 bis 13 transformiert sind.

17. Pflanzenzellen nach einem der Ansprüche 14 bis 16, die nachweisbare Mengen an Phosphinotricin-Acetyl-Transferase produzieren.

18. Verfahren zum selektiven Schützen der Kultur einer Pflanzenart und zum selektiven Zerstören von Unkraut, umfassend das Behandeln des Feldes mit einem Herbizid, bestehend aus einem Glutamin-Synthetase-Inhibitor, worin die Zellen der Pflanzenart in ihrem Genom eine fremde Nukleotidsequenz enthalten, die ein Protein mit einer enzymatischen Aktivität kodiert, die den Glutamin-Synthetase-Inhibitor neutralisieren oder inaktivieren kann, unter der Kontrolle eines durch die Polymerasen der Zellen dieser Pflanze erkannten Promotors.

19. Verfahren nach Anspruch 18, worin die Pflanzenarten ein heterologes DNA-Fragment wie in einem der Ansprüche 1 bis 6 definiert, enthalten.

20. Verfahren nach Anspruch 18 oder 19, worin die Pflanzenart gemäß dem Verfahren nach einem der Ansprüche 7 bis 13 transformiert ist.

21. Verfahren nach einem der Ansprüche 18 bis 20, worin eine Lösung des Glutamin-Synthetase-Inhibitors auf dem Acker nach Auflauf der kultivierten Pflanzenart mehrmals, insbesondere in Zeitintervallen von etwa 20 bis 100 Tagen, angewendet wird, bis früh und spät keimendes Unkraut zerstört ist.

22. Verfahren nach einem der Ansprüche 18 bis 21, worin der Glutamin-Synthetase-Inhibitor ausgewählt ist aus einer Gruppe, die Bialaphos, Phosphinotricin oder verwandte Verbindungen umfaßt.

23. Verfahren zum selektiven Schützen einer Pflanzenart auf einem Acker vor Pilzerkrankungen, umfassend das Behandeln des Ackers mit einem Herbizid, bestehend aus einem Glutamin-Synthetase-Inhibitor, worin die Pflanzenart

in dem Genom ihrer Zellen eine heterologe DNA enthält, die einen Promotor, der durch die Polymerasen dieser Zelle erkannt wird und eine fremde Nukleotidsequenz einschließt, die für ein Protein mit einer enzymatischen Aktivität kodiert, die den Glutamin-Synthetase-Inhibitor neutralisieren oder inaktivieren kann, unter der Kontrolle des Promotors.

24. Verfahren nach Anspruch 23, worin die Pflanzenart eine heterologe DNA, wie in einem der Ansprüche 2 bis 6 definiert, enthält.

25. Verfahren nach Anspruch 23 oder 24, worin die Pflanzenart mit dem Verfahren nach einem der Ansprüche 7 bis 13 transformiert ist.

26. Verfahren nach einem der Ansprüche 23 bis 25, worin eine Lösung des Glutamin-Synthetase-Inhibitors auf dem Acker nach Auflauf kultivierter Pflanzenarten mehrmals, insbesondere in Zeitintervallen von etwa 20 bis 100 Tagen angewendet wird, bis die Pilze zerstört sind.

27. Verfahren nach einem der Ansprüche 23 bis 26, worin der Glutamin-Synthetase-Inhibitor ausgewählt ist aus einer Gruppe, die Bialaphos, Phosphinotricin und verwandte Verbindungen umfaßt.

28. Verfahren nach einem der Ansprüche 18 bis 27, worin das Bialaphos, PPT oder die verwandte Verbindung in einer Menge im Bereich von etwa 0,4 bis 1,6 kg/ha angewendet wird.

29. Verfahren nach Anspruch 28, worin das Bialaphos, PPT oder die verwandte Verbindung in einem flüssigen Träger auf eine solche Konzentration verdünnt wird, daß eine Anwendung auf dem Acker in einer Menge von etwa 2 l/ha bis 8 l/ha möglich ist.

30. Verfahren nach Anspruch 28 oder 29, das selektiv zum Schutz von Pflanzenarten, ausgewählt aus Zuckerrübe, Reis, Kartoffel, Tomate, Mais, Tabak angewendet wird.

31. Vektor zur Transformation von Pflanzenzellen, enthaltend eine heterologe DNA, die einen Promotor, der durch Polymerasen dieser Pflanzen erkannt wird, und eine fremde Nukleotidsequenz enthält, die für ein Polypeptid kodiert, das eine enzymatische Aktivität aufweist, die die Inaktivierung oder Neutralisierung eines Glutamin-Synthetase-Inhibitors verursachen kann, unter der Kontrolle des Promotors.

32. Vektor nach Anspruch 31, der eine fremde Nukleotidsequenz enthält, die für ein Polypeptid mit einer Acetyltransferase-Aktivität, insbesondere PPT- Acetyltransferase-Aktivität, kodiert.

33. Vektor nach Anspruch 32, der die Regulatorelemente umfaßt, die für die stabile Integration der fremden Nukleotidsequenz in die genomische DNA der Pflanzenzellen und Pflanzen benötigt wird.

34. Vektor nach einem der Ansprüche 31 bis 33, der ein DNA-Fragment enthält, das zum Genom eines Antibiotikum-erzeugenden Streptomyces-Stammes gehört.

35. Vektor nach einem der Ansprüche 31 bis 34, worin die fremde Nukleotidsequenz für ein Protein mit der folgenden Sequenz

X SER PRO GLU

183

ARG ARG PRO ALA ASP ILE ARG ARG ALA THR GLU ALA ASP MET PRO

228

ALA VAL CYS THR ILE VAL ASN HIS TYR ILE GLU THR SER THR VAL

273

ASN PHE ARG THR GLU PRO GLN GLU PRO GLN GLU TRP THR ASP ASP

318

LEU VAL ARG LEU ARG GLU ARG TYR PRO TRP LEU VAL ALA GLU VAL

363

ASP GLY GLU VAL ALA GLY ILE ALA TYR ALA GLY PRO TRP LYS ALA

408

ARG ASN ALA TYR ASP TRP THR ALA GLU SER THR VAL TYR VAL SER

453

PRO ARG HIS GLN ARG THR GLY LEU GLY SER THR LEU TYR THR HIS

498

LEU LEU LYS SER LEU GLU ALA GLN GLY PHE LYS SER VAL VAL ALA

543

VAL ILE GLY LEU PRO ASN ASP PRO SER VAL ARG MET HIS GLU ALA

588

LEU GLY TYR ALA PRO ARG GLY MET LEU ARG ALA ALA GLY PHE LYS

633

HIS GLY ASN TRP HIS ASP VAL GLY PHE TRP GLN LEU ASP PHE SER

678

LEU PRO VAL PRO PRO ARG PRO VAL LEU PRO VAL THR GLU ILE

723

in welcher X MET oder VAL darstellt, oder für einen Teil des Proteins kodiert, worin der Teil genügend lang ist, um die enzymatische Aktivität zu besitzen und, wenn das DNA-Fragment in Pflanzenzellen exprimiert wird, letztere gegen die herbizide Aktivität eines Glutamin-Synthetase-Inhibitors zu schützen.

36. Vektor nach Anspruch 35, der die folgende Nukleotid-sequenz umfaßt:

```
                                                    Y AGC CCA GAA

     183
CGA CGC CCG GCC GAC ATC CGC CGT GCC ACC GAG GCG GAC ATG CCG
     228
GCG GTC TGC ACC ATC GTC AAC CAC TAC ATC GAG ACA AGC ACG GTC
     273
AAC TTC CGT ACC GAG CCG CAG GAA CCG CAG GAG TGG ACG GAC GAC
     318
CTC GTC CGT CTG CGG GAG CGC TAT CCC TGG CTC GTC GCC GAG GTG
     363
GAC GGC GAG GTC GCC GGC ATC GCC TAC GCG GGC CCC TGG AAG GCA
     408
CGC AAC GCC TAC GAC TGG ACG GCC GAG TCG ACC GTG TAC GTC TCC
     453
CCC CGC CAC CAG CGG ACG GGA CTG GGC TCC ACG CTC TAC ACC CAC
     498
CTG CTG AAG TCC CTG GAG GCA CAG GGC TTC AAG AGC GTG GTC GCT
     543
GTC ATC GGG CTG CCC AAC GAC CCG AGC GTG CGC ATG CAC GAG GCG
     588
CTC GGA TAT GCC CCC CGC GGC ATG CTG CGG GCG GCC GGC TTC AAG
     633
CAC GGG AAC TGG CAT GAC GTG GGT TTC TGG CAG CTG GAC TTC AGC
     678
CTG CCG GTA CCG CCC CGT CCG GTC CTG CCC GTC ACC GAG ATC
     723
```

worin Y entweder ATG oder GTG ist.

37. Vektor nach einem der Ansprüche 31 bis 36, worin die heterologe DNA eine Nukleotidsequenz umfaßt, die ein Transitpeptid kodiert, das zwischen den Pflanzenpromotorbereich und die den Glutamin-Synthetase-Inhibitor kodierende fremde Nukleotidsequenz eingeschoben ist.

38. Vektor nach Anspruch 37, der ein Ti-Plasmid ist, das die heterologe DNA enthält.

Revendications

1. Procédé de contrôle de l'action chez les cellules végétales et les plantes comprenant de telles cellules d'un inhibiteur de la glutamine synthétase lorsque celles-ci sont mises en contact avec ce dernier, comprenant la réalisation de l'intégration stable dans l'ADN génomique desdites cellules végétales d'un ADN hétérologue comportant un promoteur reconnu par les polymérases desdites cellules végétales et une séquence nucléotidique étrangère

susceptible d'être exprimée sous la forme d'une protéine dans lesdites cellules végétales et lesdits végétaux, sous le contrôle dudit promoteur, et dans lequel ladite protéine a une activité enzymatique susceptible d'entraîner l'inactivation ou la neutralisation dudit inhibiteur de la glutamine synthétase.

2. Procédé selon la revendication 1, caractérisé en ce que le fragment d'ADN hétérologue comprend une séquence nucléotidique étrangère codant pour un polypeptide ayant une activité acétyl-transférase, notamment une activité acétyl-transférase de PPT, vis-à-vis dudit inhibiteur de la glutamine synthétase.

3. Procédé selon la revendication 1 ou 2, caractérisé en ce que la séquence nucléotidique étrangère est issue du génome d'une souche de *streptomyces* productrice d'antibiotiques ou il s'agit d'une séquence nucléotidique codant pour la même activité.

4. Procédé selon l'une quelconque des revendications 1 à 3, caractérisé en ce que l'ADN hétérologue comprend une séquence nucléotidique étrangère codant pour une protéine de la séquence suivante:

```
                                                    X SER PRO GLU
          163
ARG ARG PRO ALA ASP ILE ARG ARG ALA THR GLU ALA ASP MET PRO
          228
ALA VAL CYS THR ILE VAL ASN HIS TYR ILE GLU THR SER THR VAL
          273
ASN PHE ARG THR GLU PRO GLN GLU PRO GLN GLU TRP THR ASP ASP
          318
LEU VAL ARG LEU ARG GLU ARG TYR PRO TRP LEU VAL ALA GLU VAL
          363
ASP GLY GLU VAL ALA GLY ILE ALA TYR ALA GLY PRO TRP LYS ALA
          408
ARG ASN ALA TYR ASP TRP THR ALA GLU SER THR VAL TYR VAL SER
          453
PRO ARG HIS GLN ARG THR GLY LEU GLY SER THR LEU TYR THR HIS
          498
LEU LEU LYS SER LEU GLU ALA GLN GLY PHE LYS SER VAL VAL ALA
          543

VAL ILE GLY LEU PRO ASN ASP PRO SER VAL ARG MET HIS GLU ALA
          588
LEU GLY TYR ALA PRO ARG GLY MET LEU ARG ALA ALA GLY PHE LYS
          633
HIS GLY ASN TRP HIS ASP VAL GLY PHE TRP GLN LEU ASP PHE SER
          678
LEU PRO VAL PRO PRO ARG PRO VAL LEU PRO VAL THR GLU ILE
          723
```

dans laquelle X représente MET ou VAL, ou pour une partie de ladite protéine, dans laquelle ladite partie est d'une longueur suffisante pour présenter l'activité enzymatique de la protéine entière et, lorsqu ledit fragment d'ADN est exprimé chez les cellules végétales, pour protéger ces dernières vis-à-vis de l'activité herbicide dudit inhibiteur de la glutamine synthétase.

5. Procédé selon l'une quelconque des revendications 1 à 4, caractérisé en ce que le fragment d'ADN hétérologue comprend la séquence nucléotidique suivante:

```
                                                        GTG AGC CCA GAA
      183
CGA CGC CCG GCC GAC ATC CGC CGT GCC ACC GAG GCG GAC ATG CCG
      228
GCG GTC TGC ACC ATC GTC AAC CAC TAC ATC GAG ACA AGC ACG GTC
      273
AAC TTC CGT ACC GAG CCG CAG GAA CCG CAG GAG TGG ACG GAC GAC
      318
CTC GTC CGT CTG CGG GAG CGC TAT CCC TGG CTC GTC GCC GAG GTG
      363
GAC GGC GAG GTC GCC GGC ATC GCC TAC GCG GGC CCC TGG AAG GCA
      408
CGC AAC GCC TAC GAC TGG ACG GCC GAG TCG ACC GTG TAC GTC TCC
      453
CCC CGC CAC CAG CGG ACG GGA CTG GGC TCC ACG CTC TAC ACC CAG
      498
CTG CTG AAG TCC CTG GAG GCA CAG GGC TTC AAG AGC GTG GTC GCT
      543
GTC ATC GGG CTG CCC AAC GAC CCG AGC GTG CGC ATG CAC GAG GCG

      588
CTC GGA TAT GCC CCC CGC GGC ATG CTG CGG GCG GCC GGC TTC AAG
      633
CAC CGG AAC TGG CAT GAC GTG GGT TTC TGG CAG CTG GAC TTC AGC
      678
CTG CCG GTA CCG CCC CGT CCG GTC CTG CCC GTC ACC GAG ATC
      723
```

ou la partie correspondante de celle-ci.

6. Procédé selon l'une quelconque des revendications 1 à 4, caractérisé en ce que le fragment d'ADN hétérologue comprend la séquence nucléotidique selon la revendication 5 ayant le codon d'initiation GTG substitué par le codon d'initiation ATG.

7. Procédé de production d'une plante ou d'un matériel de reproduction de ladite plante comportant un matériel génétique hétérologue intégré de manière stable dans celle-ci et susceptible d'être exprimé chez lesdites plantes ou chez ledit matériel de reproduction sous la forme d'une protéine susceptible d'inactiver ou de neutraliser l'activité d'un inhibiteur de la glutamine synthétase, lequel procédé comprend la transformation de cellules ou de tissus desdites plantes par un recombinant à and contenant un ADN hétérologue comportant une séquence nucléotidique étrangère codant pour ladite protéine ainsi que les éléments de régulation choisis parmi ceux susceptibles d'entraîner l'intégration stable dudit and hétérologue chez lesdites cellules végétales ou lesdits tissus végétaux et de permettre l'expression de ladite séquence nucléotidique étrangère dans lesdites cellules végétales ou ledit tissu végétal, la régénération de plantes ou de matériel de reproduction desdites plantes ou les deux à partir des cellules ou des tissus végétaux transformés par ledit ADN hétérologue et, éventuellement, la réplication biologique desdites plantes ou dudit matériel de reproduction précédents ou les deux.

8. Procédé selon la revendication 7, caractérisé en ce que les cellules de départ sont transformées par un ADN recombinant contenant l'ADN hétérologue tel que défini dans l'une quelconque des revendications 2 à 6.

9. Procédé selon la revendication 8, caractérisé en ce que 1'ADN recombinant est un vecteur approprié pour la transformation des cellules de ladite plante.

10. Procédé selon l'une quelconque des revendications 8 ou 9, caractérisé en ce qu'il confère une résistance vis-à-vis des effets herbicides de Bialaphos, de PPT ou de dérivés apparentés, aux cellules végétales transformées.

11. Procédé selon l'une quelconque des revendications 7 à 10, caractérisé en ce que ledit fragment d'ADN hétérologue comprend une séquence nucléotidique codant pour un peptide de transit intercalé entre ladite région de promoteur végétal et ladite séquence nucléotidique étrangère codant pour ledit inhibiteur de la glutamine synthétase.

12. Procédé selon la revendication 11, caractérisé en ce que le peptide de transit est choisi parmi la ribulose-1,5 biophosphate carboxylase et les protéines de liaison de la chlorophylle a/b.

13. Procédé selon l'une quelconque des revendications 8 à 12, caractérisé en ce que ledit vecteur est un plasmide Ti contenant ledit fragment d'ADN hétérologue.

14. Cellules végétales, transformées non biologiquement, ayant un ADN hétérologue intégré de manière stable dans leur génome, ledit ADN hétérologue contenant une séquence nucléotidique étrangère codant pour une protéine ayant une activité enzymatique non spécifique de la variété, susceptible de neutraliser ou d'inactiver un inhibiteur de la glutamine synthétase sous le contrôle d'un promoteur reconnu par les polymérases desdites cellules végétales.

15. Cellules végétales selon la revendication 14, caractérisées en ce qu'elles peuvent être régénérées en une plante susceptible de produire des graines.

16. Cellules végétales selon la revendication 14 ou 15, caractérisées en ce qu'elles sont transformées par le procédé selon l'une quelconque des revendications 7 à 13.

17. Cellules végétales selon l'une quelconque des revendications 14 à 18, produisant des quantités décelables de la phosphinotricine acétyl-transférase.

18. Procédé de protection sélective de culture d'une espèce végétale et de destruction sélective des mauvaises herbes, caractérisé en ce qu'il comprend les étapes de traitement du champ avec un herbicide constitué d'un inhibiteur de la glutamine synthétase, et en ce que les cellules des espèces végétales contiennent dans leur génome une séquence nucléotidique étrangère codant pour une protéine ayant une activité enzymatique susceptible de neutraliser ou d'inactiver ledit inhibiteur de la glutamine synthétase sous le contrôle d'un promoteur reconnu par les polymérases des cellules de ladite plante.

19. Procédé selon la revendication 18, caractérisé en ce que les espèces végétales contiennent un fragment d'ADN hétérologue tel que défini dans l'une quelconque des revendications 1 à 6.

20. Procédé selon la revendication 18 ou 19, caractérisé en ce que l'espèce végétale est transformée selon le procédé de l'une quelconque des revendications 7 à 13.

21. Procédé selon l'une quelconque des revendications 18 à 20, caractérisé en ce qu'une solution de l'inhibiteur de la glutamine synthétase est appliquée sur le champ après la levée de l'espèce végétale cultivée, plusieurs fois, notamment à des intervalles de temps d'environ 20 à 100 jours, jusqu'à ce que les mauvaises herbes germant de manière précoce ou tardive soient détruites.

22. Procédé selon l'une quelconque des revendications 18 à 21, caractérisé en ce que l'inhibiteur de la glutamine synthétase est choisi dans un groupe comprenant le Bialaphos, la phosphinotricine ou des composés apparentés.

23. Procédé de protection sélective d'une espèce végétale dans un champ contre les maladies fongiques comprenant les étapes de traitement dudit champ avec un herbicide constitué d'un inhibiteur de la glutamine synthétase, caractérisé en ce que l'espèce végétale contient dans le génome de ses cellules un ADN hétérologue comportant

un promoteur reconnu par les polymérases de ladite cellule et une séquence nucléotidique étrangère codant pour une protéine ayant une activité enzymatique susceptible de neutraliser ou d'inactiver ledit inhibiteur de la glutamine synthétase sous le contrôle dudit promoteur.

24. Procédé selon la revendication 23, caractérisé en ce que l'espèce végétale contient un ADN hétérologue tel que défini dans l'une quelconque des revendications 2 à 6.

25. Procédé selon la revendication 23 ou 24, caractérisé en ce que l'espèce végétale est transformée selon le procédé de l'une quelconque des revendications 7 à 13.

26. Procédé selon l'une quelconque des revendications 23 à 25, caractérisé en ce qu'une solution de l'inhibiteur de la glutamine synthétase est appliquée sur le champ après la levée de l'espèce végétale cultivée, plusieurs fois, notamment à des intervalles de temps d'environ 20 à 100 jours, jusqu'à ce que les champignons soient détruits.

27. Procédé selon l'une quelconque des revendications 23 à 26, caractérisé en ce que l'inhibiteur de la glutamine synthétase est choisi dans un groupe comprenant le Bialaphos, la phosphinotricine et des composés apparentés.

28. Procédé selon l'une quelconque des revendications 18 à 27, caractérisé en ce que lesdits Bialaphos, PPT ou composé apparenté sont appliqués à une dose allant d'environ 0,4 à environ 1,6 kg/ha.

29. Procédé selon la revendication 28, caractérisé en ce que lesdits Bialaphos, PPT ou composé apparenté sont dilués dans un support liquide à une concentration telle qu'elle à permette leur application sur le champ à un taux allant d'environ 2 l/ha à environ 8 l/ha.

30. Procédé selon la revendication 28 ou 29, caractérisé en ce qu'il est appliqué de manière sélective à la protection d'espèces végétales choisies parmi la betterave sucrière, le riz, la pomme de terre, la tomate, le maïs, le tabac.

31. Vecteur de transformation de cellules végétales, contenant un ADN hétérologue contenant un promoteur reconnu par les polymérases des cellules de ladite plante et une séquence nucléotidique étrangère codant pour un polypeptide ayant une activité enzymatique susceptible d'entraîner l'activation ou la neutralisation d'un inhibiteur de la glutamine synthétase sous le contrôle dudit promoteur.

32. Vecteur selon la revendication 31, caractérisé en ce qu'il contient une séquence nUcléotidique étrangère codant pour un polypeptide ayant une activité acétyl-transférase, notamment l'activité acétyl-transférase de PPT.

33. Vecteur selon la revendication 32, caractérisé en ce qu'il comprend les éléments de régulation nécessaires pour l'intégration stable de ladite séquence nucléotidique étrangère dans l'ADN génomique desdites cellules végétales et plantes.

34. Vecteur selon l'une quelconque des revendications 31 à 33, caractérisé en ce qu'il contient un fragment d'ADN appartenant au génome d'une souche de *streptomyces* productrice d'antibiotiques.

35. Vecteur selon l'une quelconque des revendications 31 à 34, caractérisé en ce que la séquence nucléotidique étrangère code pour une protéine ayant la séquence suivante:

X SER PRO GLU

183
ARG ARG PRO ALA ASP ILE ARG ARG ALA THR GLU ALA ASP MET PRO
228
ALA VAL CYS THR ILE VAL ASN HIS TYR ILE GLU THR SER THR VAL
273
ASN PHE ARG THR GLU PRO GLN GLU PRO GLN GLU TRP THR ASP ASP
318
LEU VAL ARG LEU ARG GLU ARG TYR PRO TRP LEU VAL ALA GLU VAL
363
ASP GLY GLU VAL ALA GLY ILE ALA TYR ALA GLY PRO TRP LYS ALA
408
ARG ASN ALA TYR ASP TRP THR ALA GLU SER THR VAL TYR VAL SER
453
PRO ARG HIS GLN ARG THR GLY LEU GLY SER THR LEU TYR THR HIS
498
LEU LEU LYS SER LEU GLU ALA GLN GLY PHE LYS SER VAL VAL ALA
543
VAL ILE GLY LEU PRO ASN ASP PRO SER VAL ARG MET HIS GLU ALA

588
LEU GLY TYR ALA PRO ARG GLY MET LEU ARG ALA ALA GLY PHE LYS
633
HIS GLY ASN TRP HIS ASP VAL GLY PHE TRP GLN LEU ASP PHE SER
678
LEU PRO VAL PRO PRO ARG PRO VAL LEU PRO VAL THR GLU ILE
723

dans laquelle X représente MET ou VAL, ou pour une partie de ladite protéine, dans laquelle ladite partie est d'une longueur suffisante pour présenter ladite activité enzymatique et, lorsque ledit fragment d'ADN est exprimé chez les cellules végétales, pour protéger ces dernières vis-à-vis de l'activité herbicide d'un inhibiteur de la glutamine synthétase.

36. Vecteur selon la revendication 35, caractérisé en ce qu'il comprend la séquence nucléotidique suivante:

```
                                                    Y AGC CCA GAA

          183
CGA CGC CCG GCC GAC ATC CGC CGT GCC ACC GAG GCG GAC ATG CCG
          228
GCG GTC TGC ACC ATC GTC AAC CAC TAC ATC GAG ACA AGC ACG GTC
          273
AAC TTC CGT ACC GAG CCG CAG GAA CCG CAG GAG TGG ACG GAC GAC
          318
CTC GTC CGT CTG CGG GAG CGC TAT CCC TGG CTC GTC GCC GAG GTG
          363
GAC GGC GAG GTC GCC GGC ATC GCC TAC GCG GGC CCC TCG AAG GCA


          408
CGC AAC GCC TAC GAC TGC ACG GCC GAG TCG ACC GTG TAC GTC TCC
          453
CCC CGC CAC CAG CGG ACG GGA CTG GGC TCC ACG CTC TAC ACC CAC
          498
CTG CTC AAG TCC CTG GAG GCA CAG GGC TTC AAG AGC GTG GTC GCT
          543
GTC ATC GGC CTG CCC AAC GAC CCG AGC GTG CGC ATG CAC GAG GCG
          588
CTC GGA TAT GCC CCC CGC GGC ATG CTG CGG GCG GCC GGC TTC AAG
          633
CAC GGG AAC TGG CAT GAC GTG GGT TTC TGG CAG CTG GAC TTC AGC
          678
CTG CCG GTA CCG CCC CGT CCG GTC CTG CCC GTC ACC GAG ATC
          723
```

dans laquelle Y est soit ATG soit GTG.

37. Vecteur selon l'une quelconque des revendications 31 à 36, caractérisé en ce que ledit ADN hétérologue comprend une séquence nucléotidique codant pour un peptide de transit intercalé entre ladite région du promoteur végétal et ladite séquence nucléotidique étrangère codant pour ledit inhibiteur de la glutamine synthétase.

38. Vecteur selon la revendication 37, caractérisé en ce qu'il s'agit d'un plasmide Ti contenant ledit ADN hétérologue.

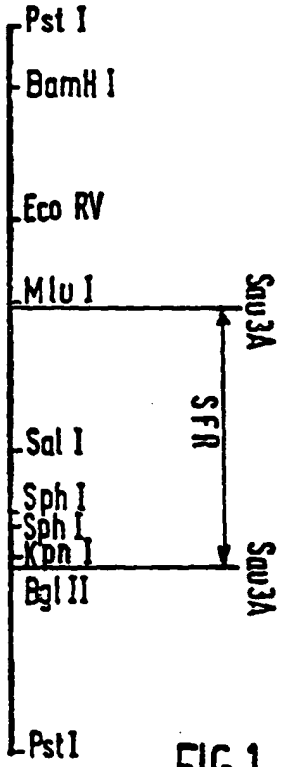
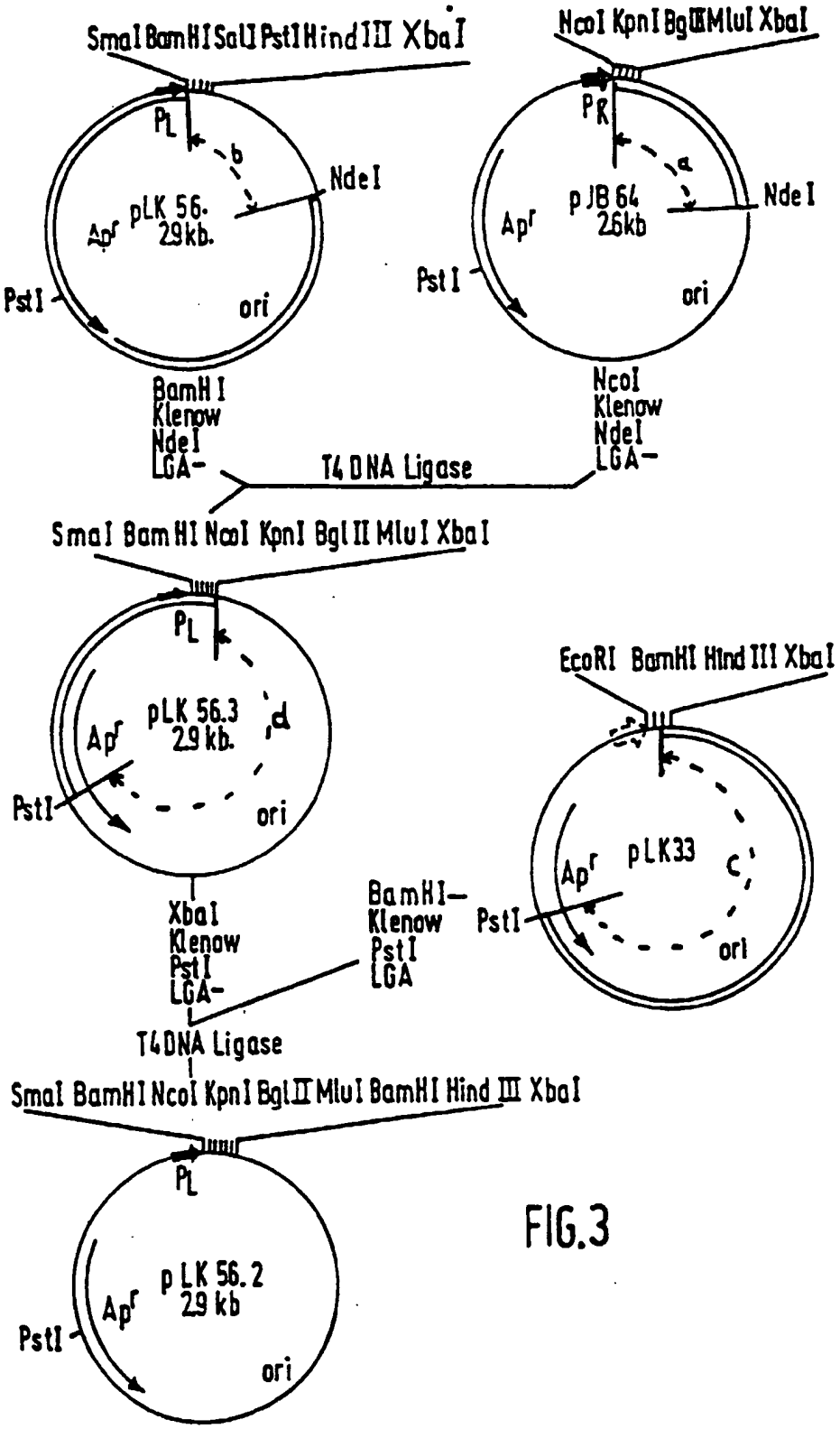

Pst I
BamH I
Eco RV
Mlu I
Sau3A
SFR
Sal I
Sph I
Sph I
Kpn I
Sau3A
Bgl II
PstI

FIG.1

CCC GCT CAA GCT CGC TGT CAT TTT CGA GAC GCC ATC TTT GGA AGC
GGT GGC CGA ATC CGT ACT GCG CGG ACT CGA CGA CGC GTA AAA CGA
TCG ACC ACG TAC ACG AGT CCG GAC ACG GGG CGA GGA GGC CCG GTT
CCG GCA CCG AGG AAG ACC GAA GGA AGA CCA CAC GTG AGC CCA GAA
CGA CGC CCG GCC GAC ATC CGC CGT GCC ACC GAG GCG GAC ATG CCG
FokI
GCG GTC TGC ACC ATC GTC AAC CAC TAC ATC GAG ACA AGC ACG GTC
AAC TTC CGT ACC GAG CCG CAG GAA CCG CAG GAG TGG ACG GAC GAC
CTC GTC CGT CTG CGG GAG CGC TAT CCC TGG CTC GTC GCC GAG GTG
GAC GGC GAG GTC GCC GGC ATC GCC TAC GCG GGC CCC TGG AAG GCA
CGC AAC GCC TAC GAC TGG ACG GCC GAG TCG ACC GTG TAC GTC TCC
CCC CGC CAC CAG CGG ACG GGA CTG GGC TCC ACG CTC TAC ACC CAC
CTG CTG AAG TCC CTG GAG GCA CAG GGC TTC AAG AGC GTG GTC GCT
GTC ATC GGG CTG CCC AAC GAC CCG AGC GTG CGC ATG CAC GAG GCG
CTC GGA TAT GCC CCC CGC GGC ATG CTG CGG GCG GCC GGC TTC AAG
CAC GGG AAC TGG CAT GAC GTG GGT TTC TGG CAG CTG GAC TTC AGC
CTG CCG GTA CCG CCC CGT CCG GTC CTG CCC GTC ACC GAG ATC TGA
BglII
ACG GAG TGC GCG TGG GCA TCG CCC GAG TTG GAG CTG GTA CGG GAA
CTC ATC GAA CTC AAC TGG CAT ACC CGC AAT GGT GAG GTG GAA CCG
CGG CGG ATC GCG TAC GAC CGT GCC CAG G

FIG.2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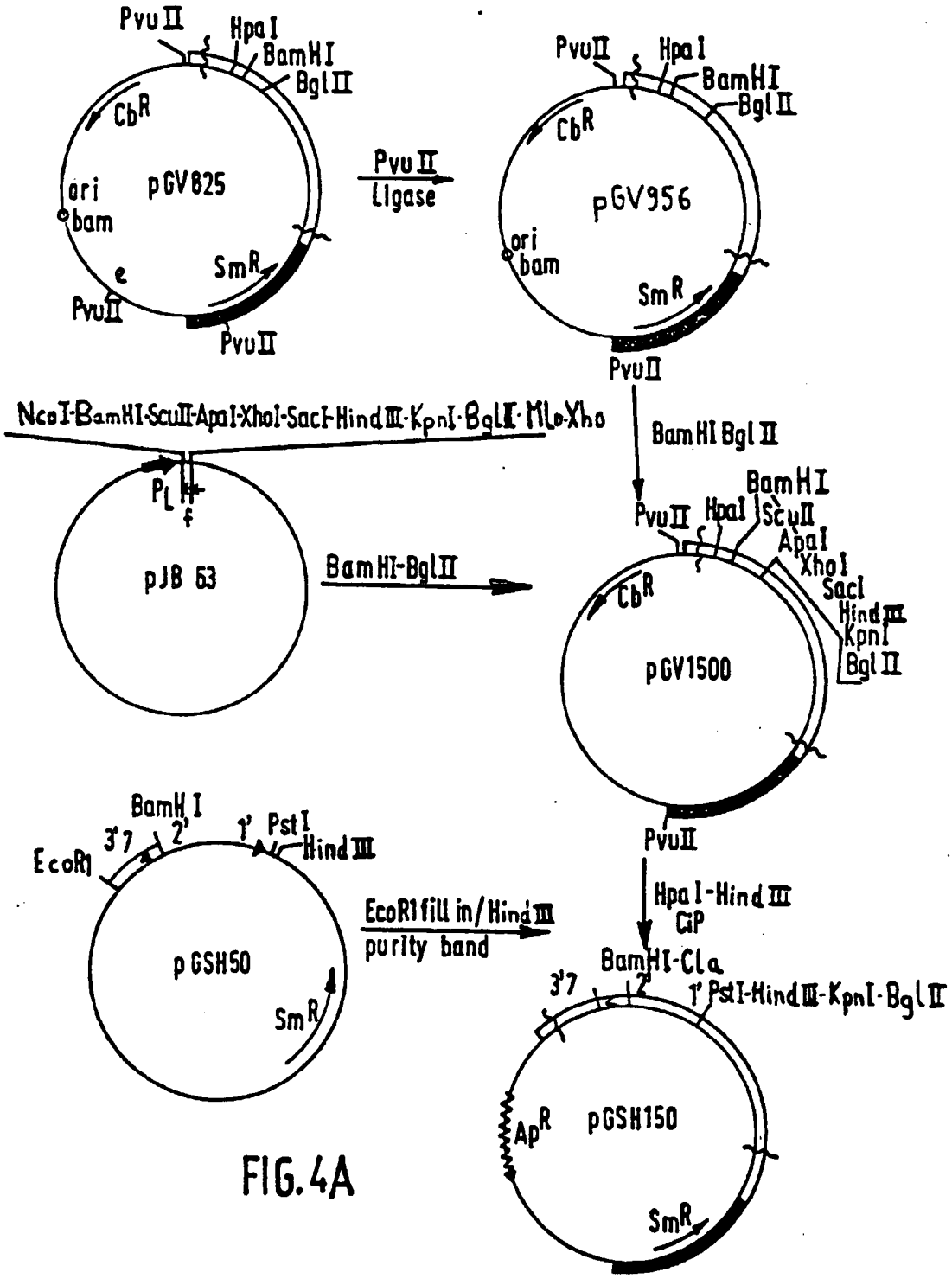
PL
b
NdeI
Apr pLK 56. 2.9kb.
PstI
ori
BamHI
Klenow
NdeI
LGA-
NcoI KpnI BglII MluI XbaI
PR
a
NdeI
Apr pJB 64 2.6kb
PstI
ori
NcoI
Klenow
NdeI
LGA-
T4 DNA Ligase
SmaI BamHI NcoI KpnI BglII MluI XbaI
PL
Apr pLK 56.3 2.9 kb.
d
PstI
ori
XbaI
Klenow
PstI
LGA-
EcoRI BamHI HindIII XbaI
Apr pLK33
c
ori
BamHI-
Klenow
PstI
LGA
PstI
T4DNA Ligase
SmaI BamHI NcoI KpnI BglII MluI BamHI HindIII XbaI
PL
Apr pLK 56.2 2.9 kb
PstI
ori

FIG.3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HpaI
BamHI
BglII
CbR
pGV825
ori
bam
e
SmR
PvuII
PvuII
PvuII
HpaI
BamHI
BglII
CbR
pGV956
ori
bam
SmR
PvuII
PvuII Ligase
NcoI-BamHI-ScuII-ApaI-XhoI-SacI-HindIII-KpnI-BglII-MluI-XhoI
BamHI BglII
PL
f
pJB 63
BamHI-BglII
BamHI
PvuII
HpaI
ScuII
ApaI
XhoI
SacI
HindIII
KpnI
BglII
CbR
pGV1500
PvuII
BamHI
3'
2'
1'
PstI
HindIII
EcoRI
pGSH50
SmR
EcoRI fill in/HindIII purity band
HpaI-HindIII CiP
BamHI-ClaI
3'
2'
1'
PstI-HindIII-KpnI-BglII
ApR
pGSH150
SmR

FIG. 4A

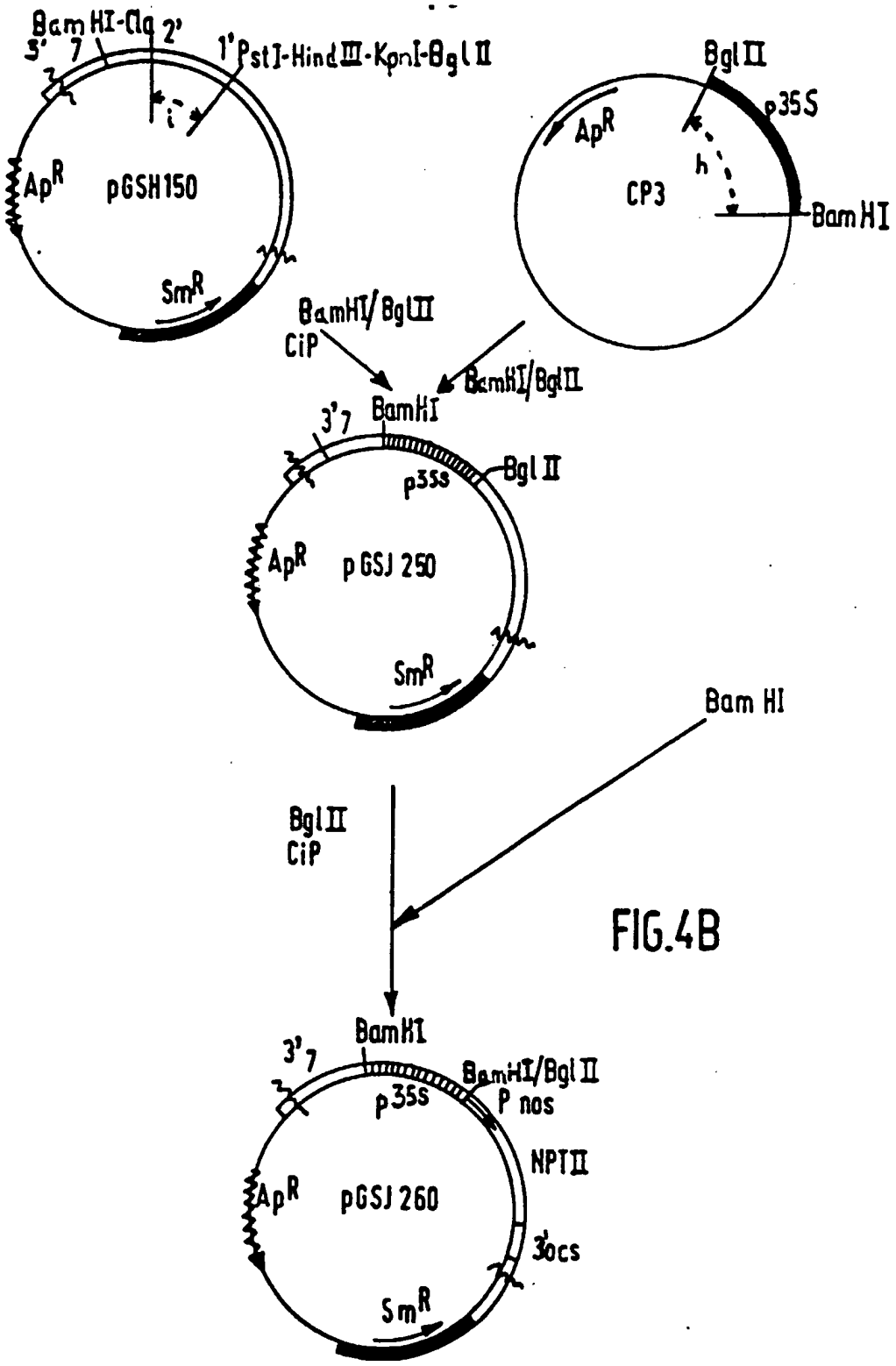

BamHI-Cla
5'
3'
2'
1'
PstI-HindIII-KpnI-BglII
ApR
pGSH150
SmR
BglII
p35S
ApR
CP3
BamHI
BamHI/BglII
CiP
BamHI/BglII
3'
5'
BamHI
p35s
BglII
ApR
pGSJ 250
SmR
Bam HI
BglII
CiP
BamHI
3'
5'
BamHI/BglII
p35s
P nos
NPTII
ApR
pGSJ 260
3'ocs
SmR

FIG.4B

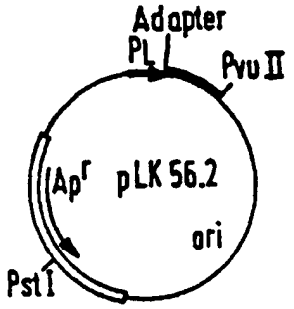


FIG.5A

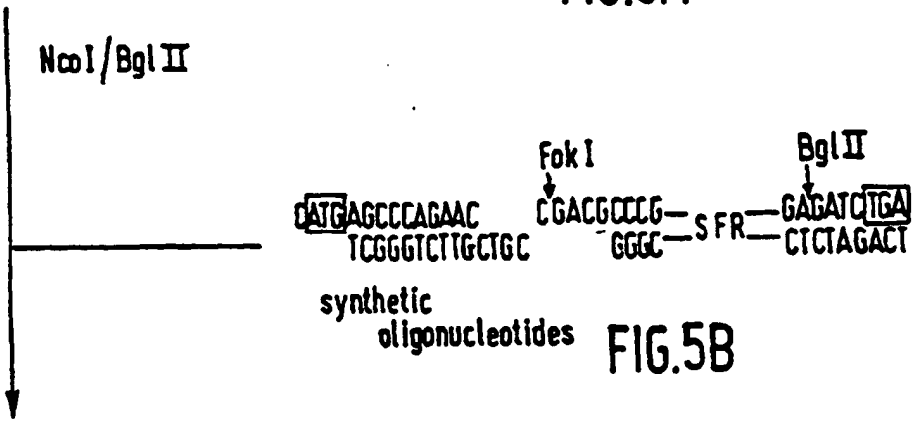


FIG.5B

FIG.5C

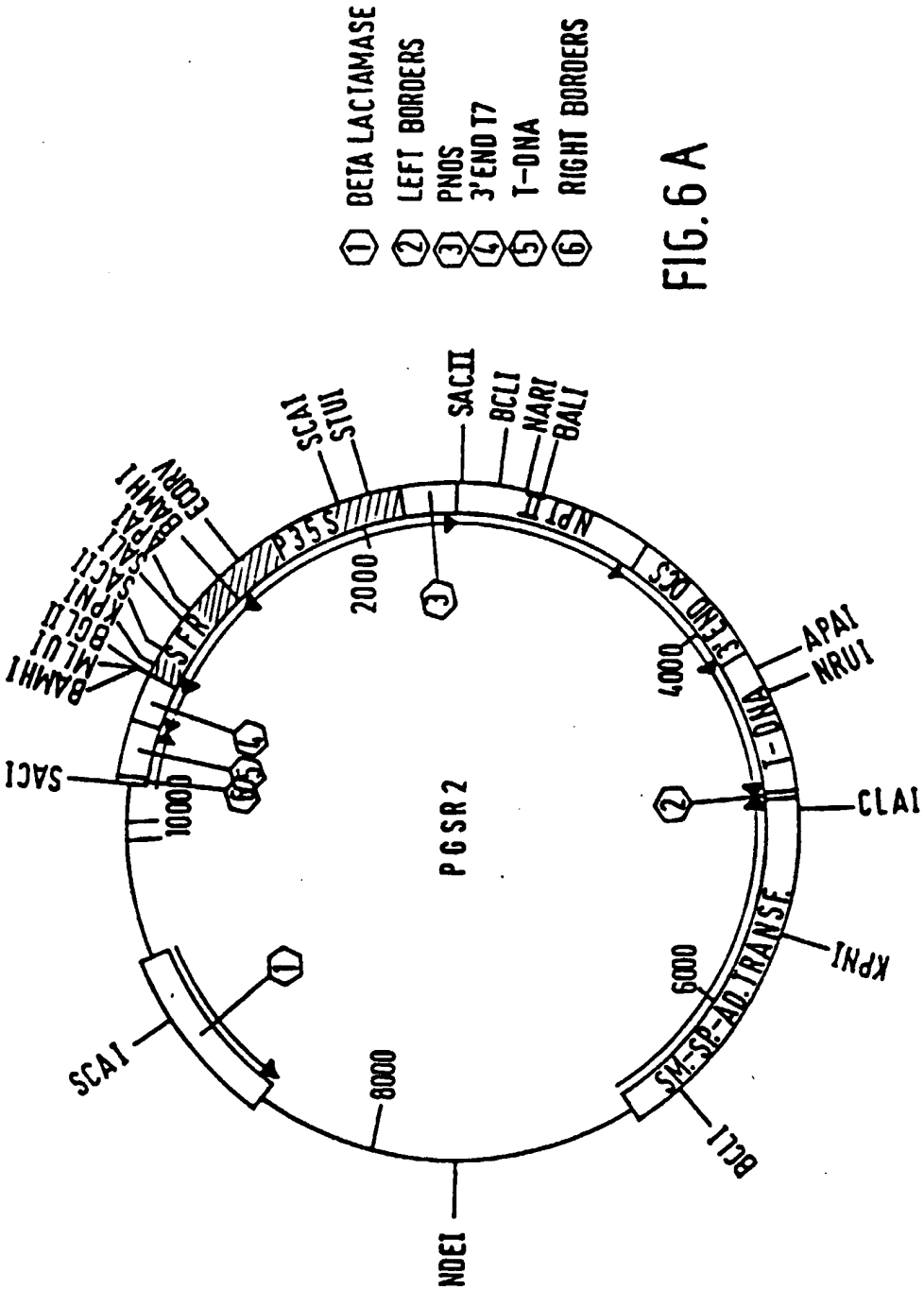
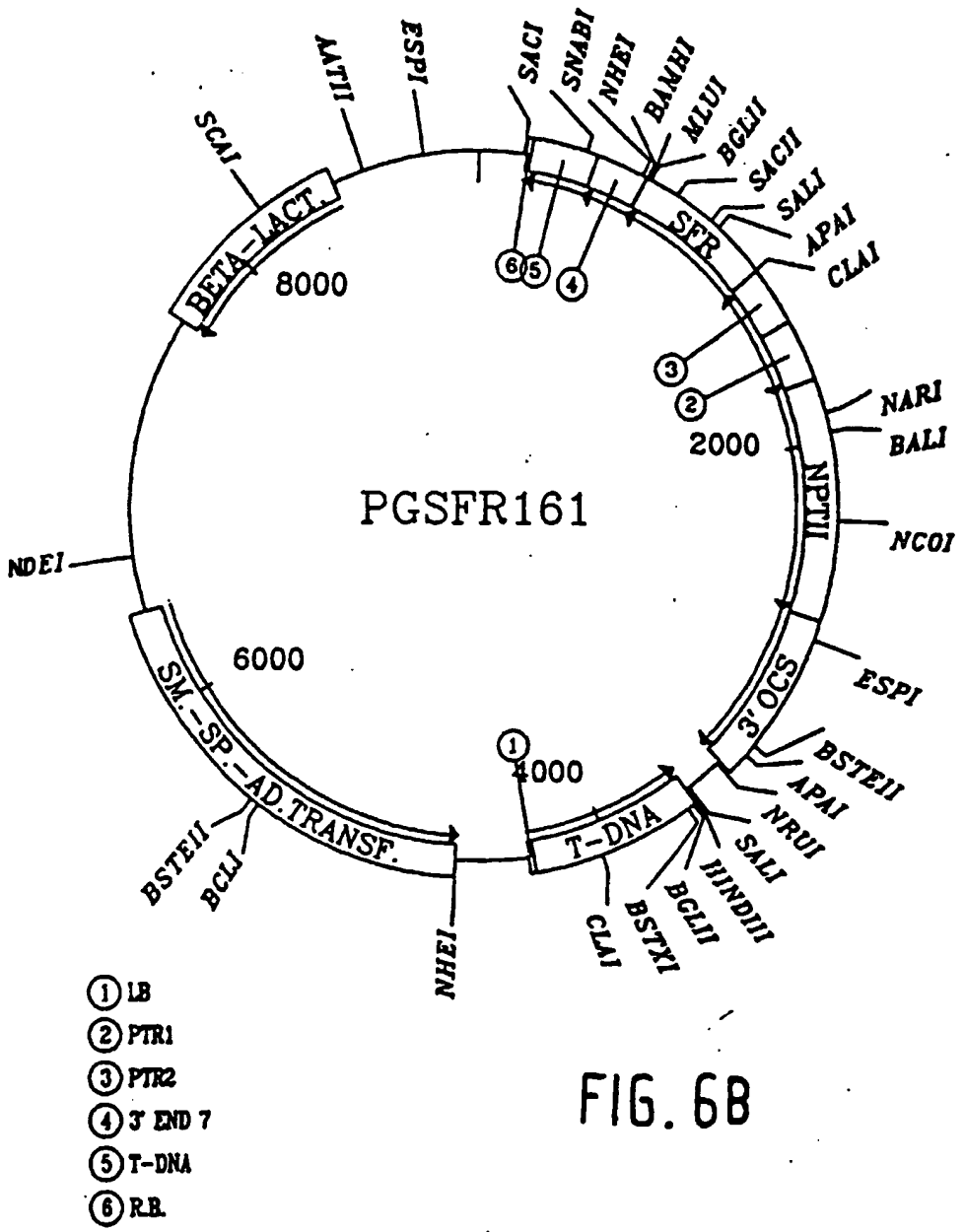

① BETA LACTAMASE
② LEFT BORDERS
③ PNOS
④ 3'END T7
⑤ T-DNA
⑥ RIGHT BORDERS
SCA1
STU1
SACII
BCLI
NARI
BALI
NPTII
3'END OCS
T-DNA
APAI
NRUI
CLAI
KPNI
BCLI
NDEI
SCA1
SACI
BAMHI
MLUI
BGLII
XPNI
SACII
SPAI
BAMHI
ECORV
P35S
SFR
2000
4000
6000
8000
10000
PGSR2

FIG. 6 A


PGSFR161
8000
2000
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6000
BETA-LACT.
SFR
NPTII
3'OCS
T-DNA
SM.-SP.-AD.TRANSF.
SCAI
AATII
ESPI
SACI
SNABI
NHEI
BANHI
MLUI
BGLII
SACII
SALI
APAI
CLAI
NARI
BALI
NCOI
ESPI
BSTEII
APAI
NRUI
SALI
HINDIII
BGLII
BSTXI
CLAI
NHEI
BCLI
BSTEII
NDEI
1 LB
2 PTR1
3 PTR2
4 3' END 7
5 T-DNA
6 R.B.

FIG. 6B

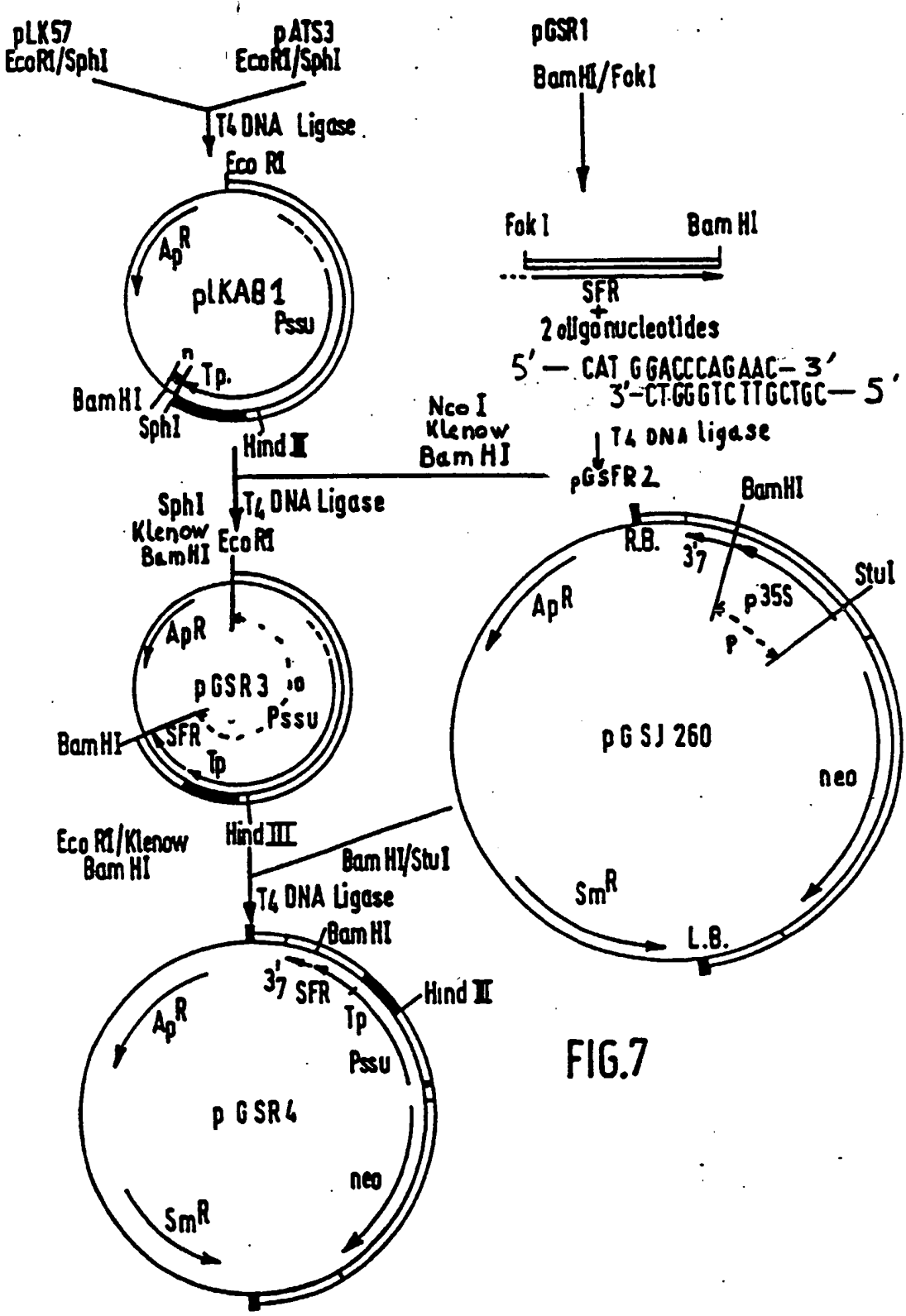
pLK57
EcoRI/SphI
pATS3
EcoRI/SphI
T4 DNA Ligase
Eco RI
ApR
plKA81
Pssu
Tp.
BamHI
SphI
Hind III
pGSR1
BamHI/FokI
Fok I
Bam HI
SFR
+
2 oligonucleotides
5'— CAT GGACCCAGAAC— 3'
3'-CT GGGTCTTGCTGC— 5'
Nco I
Klenow
Bam HI
T4 DNA ligase
pGSFR2
SphI
Klenow
BamHI
T4 DNA Ligase
Eco RI
ApR
pGSR3
Pssu
BamHI
SFR
Tp
Hind III
Eco RI/Klenow
Bam HI
Bam HI/StuI
T4 DNA Ligase
BamHI
BamHI
R.B.
3'7
StuI
ApR
p35S
pGSJ260
neo
SmR
L.B.
3'7 SFR
Hind III
ApR
Tp
Pssu
pGSR4
neo
SmR

FIG.7